LACTOBACILLUS AS A VACCINE VEHICLE

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List of Abbreviations (in alphabetical order)

APC	Allophycocyanin
BAL	Bronchoalveolar lavage
BALT	Bronchus Associated Lymphoid Tissue
BMDC	Bone marrow-derived dendritic cells
BMN	Bone marrow neutrophils
BCG	Bacillus Calmette-Guerin
BLG	Beta lactoglobulin
BSA	Bovine serum albumin
Ccl	Chemokine (C-C motif) ligand
CD	Cluster of Differentiation protein
CFU	Colony forming unit
CLN	Cervical lymph node
CMI	Cellular mediated immune (response)
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
CTLL-2	Cytotoxic T lymphocyte cell line
Cxcl	Chemokine (C-X-C motif) ligand
DAB	3, 3'-diaminobenzidine
DAPI	4, 6-diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle-Associated Epithelium
FBS	Foetal bovine serum
Fcγr1	Fc gamma receptor 1
FITC	Fluorescent isothyocyanate
GALT	Gut Associated Lymphoid Tissue
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
H & E	Hematoxylin & Eosin Staining
HPV	Human papilloma virus
HRP	Horseradish peroxidise
IACUC	Institutional Animal Care and Use Committee
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL-	Interleukin
IP-10	Interferon-inducible protein 10
KLK3	kallikrein-related peptidase 3
LAB	Lactic Acid Bacteria
ldh	lactate dehydrogenase
LGG	Lactobacillus rhamnosus GG
LP	Lamina Propria
MALT	Mucosa Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
MLN	Mesenteric Lymph Nodes
MRS	de Man, Rogosa, Sharpe
NALT	Nasopharyngeal Associated Lymphoreticular Tissue
NK	Natural Killer cells

List of Abbreviations (continued)

NUS	National University of Singapore
PA	Protective antigen
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pgm	Phosphoglyceromutase
pIgR	Polymeric Immunoglobulin Receptor
PMN	Polymorphonuclear cells
PP	Peyer's patches
PPR	Pattern Recognition Receptors
PSA	Prostate Specific Antigen
RANK	Receptor Activator of NF-κb
RBC	Red blood cell
RBS	Ribosome binding site
RT	room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SlpA	Surface layer protein A
TAM	TYRO3, AXL and MER
TBS	Tris Buffered Saline
TCR	T cell receptor
TEM	Transmission electron microscopy
TGF-β	Tumour Growth Factor-beta
Th1	Helper T cell responses 1
TLR	Toll-like receptor
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNFβ	Tumor necrosis factor β
TT	Tetanus toxoid
TTFC	Tetanus Toxin Fragment C
UEA	Ulex europaeus agglutinin
UTLS	untranslated leader sequence
WGA	Wheat germ agglutinin

Publications

1. Matheswaran Kandasamy, Anita Selvakumari Jayasurya, Shabbir Moochhala, Boon Huat Bay, Yuan Kun Lee and Ratha Mahendran. Co-delivery of IL-2 and an antigen, the green fluorescence protein, by *Lactobacillus rhamnosus* GG results in increased CD8 and CD4 T cells responses (under revision).

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Conference Papers - Poster presentation

1. A study of the internalization of Lactobacillus rhamnosus GG in the intestines of mice by transmission electron microscopy
   Conference name: Jahre Deutsche Gesellschaft für Zellbiologie – Jahrestagung. No. of Abstract: O-33, Organizer/Publisher: European Journal of Cell Biology, Heidelberg, Germany. Date: 16-19, March 2005.

2. Mucosal immunization with Lactobacillus rhamnosus GG expressing green fluorescent protein and interleukin-2 augments specific antibody production. Conference name: 1st Joint Meeting of European National Societies of Immunology. No. of Abstract: PD-2779, Organizer/Publisher: European Federation of Immunological Societies, Paris, France.
   Date: 6 -9, September 2006.

3. Lactobacillus mediated mucosal delivery of IL2-GFP fusion protein enhanced specific CTL response. Conference name: AACR Centennial Conference –Translational Cancer Medicine: Technologies to Treatment.
   No. of Abstract: A10, Organizer/Publisher: American Association of Cancer Research, Singapore, Date: 4-8, November 2007.

4. Producing LGG secreting PSA and PSA plus cytokines IL2 or IL7 or IL15 to assess their effect on the development of an antigen specific

immune response to PSA. Conference name: The 5th Asian Conference on Lactic Acid Bacteria: Microbes in Disease Prevention & Treatment. No. of Abstract: P27, Organizer/Publisher: Asian Federation of Societies for Lactic Acid Bacteria, Singapore, Date: 1-3, July 2009.

Summary

Lactobacilli are attractive candidates for vaccine delivery vehicles because they are considered as GRAS (Generally regarded as safe) organisms with a very long record of safe oral consumption. They have greater intrinsic immunogenicity and colonizing ability in the GI tract that make them potentially better candidates for vaccination. The health promoting effects of *Lactobacillus rhamnosus* GG have also been studied extensively; however it has been poorly exploited as a vaccine delivery vehicle. This dissertation aims to characterize LGG as vaccine delivery vehicle. Mucosal immunization with LGG expressing GFP or IL2-GFP induced GFP specific serum IgG and IgA. The fusion of IL2 to GFP resulted in significantly increased GFP specific serum IgA and IgG and SIgA titers compared to LGG-GFP immunization. Immunization in nasal route showed no abnormal lung damage though increased cellular infiltration was seen initially and subsequently reverted close to normal. Immunohistochemical staining of the lung tissue showed IgA producing B cells at 80^{th} day of post primary immunization. There were increased GFP specific CD8 T cells in the recall assay which was significantly increased by IL2- GFP mucosal delivery.

Members of γc cytokine family (IL7, IL15 and IL2) have been expressed with PSA in LGG and co-cultured *in vitro* with DC or neutrophils to study the antigen presentation. LGG itself have stimulatory effects on DC maturation and increased the expression of CD86, CD80, CD40 and MHC II. IL15-PSA or IL2–PSA secreting LGG reduced IL10 production by DC, IL7 did not, but all three resulted in increased IL12p70 production. However, the T cell response did not correlate with differences in IL12 or IL10 production.

LGG-S-IL15-PSA treated DC primed T cells showed high IFNγ production and CTL response on target cells indicating efficient antigen presentation to T cells. LGG treated neutrophils did not induce any of the co-stimulatory molecules or MHC II expression but only showed elevated expression of the MHC I molecules. LGG treated neutrophils produced high and moderate levels of IL10 and IL12p70 respectively and efficiently induced allogeneic T cell proliferation. LGG treated neutrophils increased the expression of co-stimulatory molecules on DC that clearly showed bacteria treated neutrophils could deliver the maturation signals to immature DC. Recombinant LGG treated neutrophils provided antigen specificity to DC by unknown mechanism when it was co-cultured with DC and also rendered a cytotoxic effect in T cell presentation. This ensures the efficacy of LGG based antigen delivery in inducing immune response through neutrophils alone in the absence of direct bacteria-DC encounter. This dissertation showed that LGG as a promising antigen delivery vehicle and that IL15 is a good vaccine adjuvant especially when administered as fusion protein with antigen.

Chapter one
Introduction

1.1. Mucosal immune system - An Overview

The mammalian mucosal immune system consists of a network of lymphoid tissues which frequently encounters foreign invaders at mucosal surfaces. The mucosal surface is the major portal of entry for infectious agents and it has a vast and enormous surface area, approximately 300 to 400 m^2. This requires a formidable defence system mainly contributed by the Mucosa Associated Lymphoid Tissue (MALT) through secretory IgA and effector T cells that act synergistically with the innate immune system (Fujihashi *et al.* 2008). The mucosa contains the highest lymphocyte concentration, approximately about 6 x 10^{10} antibody-forming cells in MALT compared to 2.5 x 10^{10} lymphocytes in the lymphoid organs. The main components of MALT are Gut Associated Lymphoid Tissue (GALT), Bronchus Associated Lymphoid Tissue (BALT) and Nasopharyngeal Associated Lymphoreticular Tissue (NALT). The GALT is comprised of the Peyer's patches (PP), the appendix, and the solitary lymphoid nodules. The tonsils and adenoids (human) or nasal associated lymphoreticular tissue comprise the NALT (Staats *et al*. 1996).

Most human pathogens enter the body through a mucosal surface, such as the intestine, and strong immune responses are required to protect this physiologically essential tissue. However active immunity against non-pathogenic materials would be dangerous and lead to inflammatory disorders such as Coeliac disease and Crohn's disease. As a result, the usual response to harmless gut antigens is the induction of local and systemic immunological tolerance, known as oral tolerance (Strobel *et al*. 1998). The intestinal microflora play important roles in the modulation of oral tolerance (Moreau and Corthier *et al*, 1988). Administration of probiotics could restore oral tolerance in germfree

mice, and those effects are strain-dependent (Maeda *et al*. 2001). Immune tolerance in Lactobacilli administration may be avoided by choosing specific strain that induces Th1 rather than Th2 immune response (Drago *et al*. 2010)

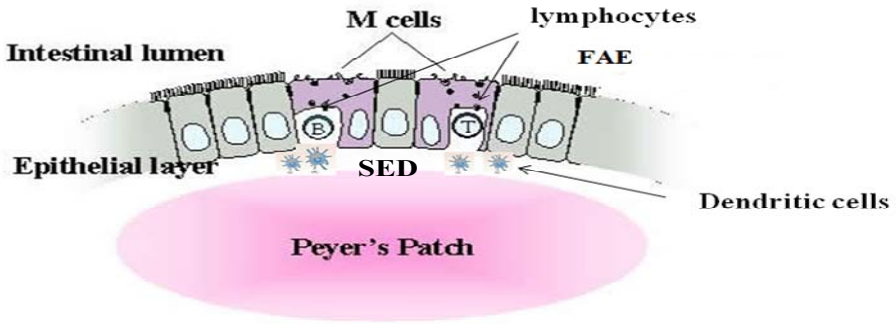


Figure 1.1 Schementic representation of gut associated lymphoid tissue (GALT)

Peyer's patches are composed of a specialized follicle-associated epithelium (FAE) containing M cells, a subepithelial dome (SED) rich in dendritic cells (DCs), B and T lymphocytes.

1.1.1. Peyer's patches (PP)

The PP germinal centres in the gastrointestinal tract are the major sites for frequent B cell switches to IgA (Lebman *et al*. 1987; Butcher *et al.* 1982). Peyer's patches are one of the major sources of IgA plasma cell precursors that undergo direct antigen driven proliferation. After antigenic stimulation, IgA^+ lymphoblasts migrate through the lymph and blood circulation and eventually

home in to the lamina propria of the intestine. Mature Peyer's patches consist of collections of large B-cell follicles and intervening T-cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated epithelium (FAE) and the most notable feature of FAE is the presence of microfold cells (M) cells, which are specialized enterocytes that lack surface microvilli and the thick layer of mucus (Mowat *et al.* 2003). M cells serve as portals of entry for pathogens (Jones *et al.* 1994) and are known to internalize and transport luminal antigens into the underlying lymphoid tissue (Wolf and Bye *et al.* 1984) where antigen presenting cells will acquire the antigens and present them to T cells after processing.

1.1.2. Intestinal enterocytes

Intestinal enterocytes are also known to process and present antigens (Zimmer *et al.* 2000), but they only induce tolerance since they do not express the co-stimulatory molecules that are required for full T cell activation (Sanderson *et al.* 1993). However intestinal epithelial cells were reported to express non classical restriction elements CD1 and T1 in mouse and CD1d in man (Bleicher *et al.* 1990; Blumberg *et al.* 1991; Panja *et al.* 1993). These class Ib molecules are capable of binding peptides and interestingly non-peptide antigens. Intestinal mucosa can discriminate pathogenic and non-pathogenic bacteria which may depend on recognition by pattern recognition receptors (PPR). Intestinal epithelial cells secrete fluid in response to invasive bacteria (Eckmann *et al.* 1997). For non pathogenic bacteria 2 models have been proposed to explain the intestinal epithelial cell response. In the first model, Gram negative *Escherichia coli* and certain *lactobacilli* could trigger a NF-κB mediated inflammatory

response which is transient and is suppressed by immune cells which predominantly secrete IL-10 in the lamina propria. A second type of response is triggered by commensals that do not induce a pro-inflammatory response, but evoke the activation of TGF-β which induces tolerance and protects barrier integrity (Schiffrin *et al.* 2002). Recently a receptor mediated antigen uptake mechanism involving fetal Fc receptor has been established (Baker *et al.* 2009). This receptor binds IgG by a pH sensitive mechanism that facilitates vesicular bidirectional transport of intact-IgG or IgG-antigen complexes across mucosal epithelial cells and delivers them to underlying DCs to initiate T cell response and alternatively it can deliver IgG antibodies to the mucus lumen for the purpose of host defense against epithelial cell-associated pathogens (Yoshida *et al.* 2006).

1.1.3. Mesenteric lymph nodes (MLN)

The MLNs are the largest lymph nodes in the body. It is considered as the cross roads between the peripheral and mucosal recirculation pathways (systemic immune system). Antigen encountered DCs prime the T cells in PP and exit through the draining lymphatics to the MLN or prime the T cells in MLN and reside for an undefined period for further differentiation. Then they migrate into the blood stream through the thoracic duct and finally accumulate in the mucosa to give efficient local immune response or tolerance (Mowat *et al.* 2003).

1.1.4. Mucosal dendritic cells

Several subsets of DC have been identified in the PP (Iwasaki *et al.* 2000; Johansson *et al.* 2005; Kelsall *et al.* 2005). In addition to myeloid ($CD8\alpha^-$

$CD11b^+$) and lymphoid ($CD8\alpha^+$ $CD11b^-$) subsets another subset ($CD8\alpha^-$ $CD11b^-$) was also found at the dome region immediately beneath the FAE. The distinguishable feature of the DC subsets in PP is their ability to secrete IL-10 rather than IL-12 which is produced by splenic DC in response to activation after ligation of the co-stimulatory molecule receptor activator of NF-κb (RANK) (Williamson *et al.* 2002).

DCs at lamina propria (LP) process the antigen delivered by intestinal enterocytes or internalize the organism by extending their cellular processes into the lumen after migrating to the epithelial monolayer in the presence of bacteria. After processing the antigen, lamina propria DC interact with T cells mainly at the MLN rather than in the mucosa itself (Mowat *et al*. 2003).

In antigen fed mice, DCs in the MLN produce IL-10 or TGFβ and preferentially stimulate antigen specific $CD4^+$ T cells to produce IL-10 and or TGF-β (Akbari *et al.* 2001). This T_R1 or T_H3 cytokine pattern has been implicated in oral tolerance (Groux *et al.* 1997).

1.1.5. Mucosal lymphocytes

The major population of T lymphocytes in intestines are lamina propria T cells, intraepithelial T cells and PP T cells. Lamina propria T cells and intraepithelial T cells express different pattern of T cell receptor (TCR) and TCRγδ cells are distinguished from their TCRαβ cell counterparts by their distinct set of somatically rearranged variable (V), diversity (D), joining (J), and constant (C) genes. The vast majority of T cells express a TCR composed of an alpha chain and a beta chain, whereas a minor T-cell population is characterized by the TCR gamma/delta. In contrast to conventional alpha/beta T cells, which are specific

for antigenic peptides presented by the major histocompatibility complex, gamma/delta T cells directly recognize proteins and even nonproteinacious phospholigands. In mice duodenum and jejunum, the intraepithelial T cells are present in higher numbers than lamina propria T cells and the distribution of TCRαβ cells to TCRγδ cells in intraepithelium is 1:3. In ileum it is reversed to 3:1. But the proportion of TCRαβ cells to TCRγδ cells in lamina propria is consistent throughout the small intestine at 3:1 (Tamura *et al.* 2003). The major distribution of TCRαβ cells in lamina propria and the ability of antigen recognition by TCRαβ-MHC molecule interaction make lamina propria the main site for immune responses to be executed (Tamura *et al.* 2003). The nonclassical class I MHC (class Ib) molecules are recognized by TCRγδ cells and TCRγδ has a potential antiviral immune function (Sciammas *et al.* 1999). Interaction among TCRγδ, $CD4^+$ TCRαβ and IgA B cells is reported to be necessary for maximum IgA responses (Kiyono *et al*. 1996)

1.2. Mucosal Vaccines

Injected vaccines are generally poor inducers of mucosal immunity and therefore less effective against mucosal surface infections unlike mucosally administered vaccines (Levine *et al.* 2000; Lamm *et al.* 1997). Mucosal vaccines induce a humoral response at the site of pathogen entry, are easy to administer without the need for sterile needles and syringes and thus have the potential for easy mass immunization. An important characteristic of the mucosal immune response is the local production and secretion of dimeric or multimeric immunoglobulin A which are resistant to degradation at the protease rich mucosal surfaces. Secretory IgA entraps the antigen or pathogenic microorganism and intercept

polymeric immunoglobulin receptor (pIgR) mediated pathogen transport (Lamm *et al*. 1997)

The antigen specific B cell response to mucosally delivered vaccines is dependant on $CD4^+$ Th cells and the frequency of Th1 and Th2 cell responses. In particular Th1 cells secreting IFNγ, IL2, and tumor necrosis factor β (TNFβ) are less efficient in antibody induction than the Th2 subset. In the murine system, Th1 cells through the secretion of IFNγ are more efficient in the stimulation of IgG2a production, whereas Th2 cells producing IL-4 induce IgG1 and IgE antibodies (Snapper *et al.* 1988; Finkelman *et al.* 1989). The type of immune response induced by immunization determines the efficacy of the vaccine. For example, cellular mediated immune responses (CMI) clear intracellular pathogens whereas strong antibody responses may be preferable to neutralize the effect of bacterial toxins. Vaccine adjuvants or cytokines may be co-administered to induce the desired immune response.

However, with mucosal vaccines the concentration of antigen delivered and absorbed in the body or bioavailability of the antigen is poorly characterized. Hence, only a few mucosal vaccines have been approved for human use namely, oral vaccines against polio virus (Modlin *et al.* 2004), *Salmonella typhi, Vibrio cholerae* (Levine *et al.* 2000), rota virus (Kapikian *et al.* 1996) and nasal vaccines against the influenza virus (Belshe *et al.* 1998).

The success of mucosal immunization is determined by the following factors:

1) Effective delivery of antigen,

2) Enhancement of mucosal immune response with immunomodulators or adjuvants

3) Choice of a regime and

4) Route of mucosal immunization.

Mucosal vaccine- challenges

In recent years, the use of mucosal vaccines have been given more attention. Despite much progress, there are several issues that still need to be addressed.

1. Mucosal vaccines that are administered orally or intranasally get diluted in mucosal secretion and trapped in mucus gel. Relatively larger doses of vaccine may be required and it is hard to determine what dose actually crosses the mucosal layer.

2. The most frequently asked question about the mucosal vaccine is the possible induction of mucosal tolerance. It is known that repeated oral ingestion of antigen results in decreased or totally abrogated responsiveness to subsequent systemic immunization with the same antigen. Though some mucosal vaccines are intrinsically immunogenic, additional adjuvants like cholera toxin (CT) or cytokine co-expression may help to avoid tolerance induction.

3. Recombinant bacterial vaccines when administered induce immune responses against the vaccine antigen initially, but the response to the neo-antigen is overwhelmed by response to the more immunodominant antigen of the bacteria itself.

4. The effectiveness of the live bacterial vaccines is partly dependant on the transport to organised lymphoid tissues. Vaccines derived from pathogenic microbes like live attenuated *S. typhi* (Levine *et al.* 2000) or live attenuated polio virus (Modlin *et al.* 2004) preferentially adhere to M cells and exploit M-cell transport to invade organized mucosal lymphoid tissues in the intestines (Jones *et al.* 1994; Sicinski *et al.* 1990). *Lactobacilli* are not known to invade lymphoid

tissue as the microbes discussed above. However expressing foreign protein with DC targeting peptide may improve bacterial uptake (Mohamadzadeh *et al.* 2009).

5. The important challenge is to apply the results obtained with animal studies to clinical trials and to moitor systematically all parameters of the immune response.

1.2.1. Live bacterial vaccines

Both attenuated pathogenic bacteria and commensal microorganisms have been successfully used as carriers for vaccine antigens (Thole *et al*, 2000). Live attenuated pathogens have the double advantage that they provide protective immunity to the pathogen and also elicit specific immune responses for the heterologous antigen that is carried by the pathogen. They also usually colonize the mucosae, ensuring prolonged exposure to the immune system for effective priming. Thus they do not necessitate repeated administrations. *Listeria monocytogenes, Salmonella* spp., *V. cholera, Shigella* spp., *Mycobacterium bovis* BCG and *Yersinia enterocolitica* are successfully used as attenuated mucosal pathogens in animal models. Table 1.1 shows the list of some attenuated pathogenic bacteria used as vaccine vehicles. Commensal microorganisms, *Streptococcus gordonii, Lactobacillus* spp. and *Staphylococcus* spp. are also commonly used as antigen delivery systems (Medina *et al.* 2001).

1.2.2. Disadvandages of using attenuated pathogenic bacteria as vaccines

1. A potential risk of reversion to virulence.

2.. Doses effective in non-endemic areas may not be effective in endemic areas where normal wild type strains are circulating (Detmer *et al.* 2006).

3. Immune induction against the antigen expressed in live bacteria may be compromised if the host has pre-existing immunity against carrier strain (example - *Salmonella*).

4. Permanent colonization of the intestines by the vaccine carrier may result in gene/plasmid transfer to the host's indigenous flora competitive exclusion of indigenous flora .

Table 1.1. Attenuated pathogenic bacteria as vaccine vehicles

Microorganisms	Mechanism of immune induction	Advantages	Disadvantages	References
Listeria monocytogenes (mutated in virulence associated determinants)	MHC class I restricted immune response	Elicits strong cellular response. Useful in clearance of intracellular pathogens and cancer. *Listeria* based vaccine is under clinical trial phase I/II.	If virulence is not severely attenuated or if the mutated strain reverts to normal, listeria infection causes a fatal disease called listeriosis.	Jiang *et al.* 2007, Bruhn *et al.* 2007.
Salmonella spp (mutants deficient in the biosynthesis of aromatic aminoacids or purines or cAMP, mutations affecting the global regulatory system phO/phQ have been used)	MHC class I and class II restricted immune response	Induce strong humoral and cellular immune response. Useful in viral diseases and cancer	Possibility for conversion from avirulent to virulent and translocation to organs.	Bumann *et al.* 2001, Medina *et al.* 2001, Detmer *et al.* 2006.
Yersinia enterocolitica (Yop mutant, attenuated strain WA-314 *sodA*)	MHC class I restricted antigen presentation	Induce cellular immune response. Useful against viral diseases and cancer.	Poor colonizing ability in intestine and sometimes failure to elicit $CD8^+$ T cell response in vivo.	Leibiger *et.al.* 2008, Gundel *et.al.* 2003.
Shigella spp. (induced mutation in virulent plasmid to generate non invasive, *S. flexneri* 2a. Istrati T32 is claimed be safer. Invasive- Mutations in either icsA and/or in a variety of metabolic genes)	elicits $CD8^+$ T cell response.	Deliver DNA vaccine plasmids to mucosal sites and induce protective T cell responses	Possibility for conversion from avirulent to virulent and translocation to organs	Shata *et al.* 2001. Vecino *et al.* 2002, Jennison *et al.* 2004.
Nasal administration of an attenuated strain of Bordetella pertussis (BPZE1) provided effective and sustained protection against lethal challenge with two different influenza A virus subtypes	An effective and sustained protection against lethal challenge with mouse-adapted H3N2 or H1N1 (A/PR/8/34) influenza A viruses	BPZE1 treatment protects mice from influenza virus-induced immunopathology and lymphocyte depletion.	The viral load is not significantly reduced in BPZE1-treated mice	Li *et al*. 2010.

1.2.3. Commensal microorganisms as vaccine vehicles

Different *Lactobacillus* spp. based vaccines have been developed and administered by the mucosal route, leading to the elicitation of both mucosal and systemic immune responses against the expressed antigens (Zegers *et al.* 1999; Gerritse *et al.* 1990*). Lactococcus lactis* (a non colonizing strain), *Lactobacilli* (which are able to colonize) and *Streptococcus gordonii*, (an oral commensal organism of human origin which has been known for its stable antigen presentation) are commonly used vaccine carriers. Recombinant *S. gordonii* has been employed to develop vaccines against sexually transmitted pathogens (Di Fabio *et al.* 1998).

1.3. Lactic Acid Bacteria as vaccine vehicles

Lactic acid bacteria (LAB) are a group of Gram positive non-sporulating bacteria that include species of *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus.* LAB are attractive candidates for vaccine delivery vehicles because they are considered as GRAS (Generally regarded as safe) organisms with a very long record of safe oral consumption. They have the following advantages as vaccine delivery vehicles (Wells *et al.* 2008).

1. LAB strains are able to effectively survive passage through the stomach. However the survival of LAB in stomach acid and contact with bile is strain dependent.

2. The mucosal route of administration can stimulate systemic immune response and elicit mucosal immune response by the induction of secretory Immunoglobulin A.

3. When administered by the oral route , LAB can be taken up into the PP, the major inductive site in the GI tract.

4. Killed recombinant LAB can be used for intranasal immunization.

5. LAB only induce a low-level immune response against themselves.

6. Colonizing LAB can synthesize the antigen continuously at the desired mucosal surface thereby triggering the underlying immune system.

If LAB are used for immunization a high level of antigen synthesis will not be a prerequisite as they can colonise the gastrointestinal tract. In the case of colonizers, strains appropriate for human use have to be selected on the basis of safety. Amongst LAB, the natural inhabitants of the gastrointestinal tract, *Lactococcus lactis, Lactobacillus* spp and colonizers of the oral cavity, *Streptococcus gordonii* are commonly used as vaccine carriers.

Cytokine co-expression with antigen in LAB based vaccines

Cytokine co-expression with antigen that delivered by LAB was believed to enhance the immune response than the antigen alone. Intranasal immunization of lactococci expressing Tetanus Toxoid Fragment C (TTFC) with either IL-2 or IL-6 resulted in a more rapid response and higher endpoint titres of TTFC-specific antibodies (Steidler *et al*, 1998). Co-administration of IL12 secreting *L. lactis* with a cell wall-anchored human papillomavirus type 16 E7 antigen elicited more antigen specific cellular immune response than E7 antigen expressing *L. lactis* administration alone (Bermudez-Humaran *et al*, 2003). IL10 secreting *L. lactis* was shown to alleviate the symptoms in Crohn's disease and Irritable Bowel Disease (IBD) (Steidler *et al*, 2000). Co-administration of IL10

secreting *L. lactis* with allergen expressing *L. lactis* may be useful in asthma like allergic disorders (Frossard *et al*, 2007).

1.3.1. *Lactococcus lactis*

Unlike other LAB, *Lactococcus lactis* does not colonize the digestive tract of man or animals. In mice it persists in digestive tract for less than 24 hours and in humans it passes through the gut in 3 days (Mercenier *et al.* 2000). As it has limited capacity to produce and secrete antigen *in vivo*, *Lactococcus lactis* has been engineered to express antigen intracellularly, so that the bacteria are pre-loaded with antigen before they are used for immunization. Using the *lactococcal* T7 system, heterologous antigens have been expressed so that they make up about 2-20% of total cellular protein (Mercenier *et al.* 2000). The most complete immunological study has been conducted with recombinant *Lactococcus lactis* producing the Tetanus Toxin Fragment C (TTFC) (Robinson *et al.* 1997; Norton *et al.* 1995; Steidler *et al.* 2002). *Lactococcus lactis* expressing TTFC on the membrane, intracellularly or as a secretory protein were administered without adjuvant to mice. All three *Lactococcal* TTFC expressor strains were able to elicit antibodies and protected the mice from lethal toxin challenge. Intranasal immunization of *Lactococcus lactis* that co-express TTFC with murine IL2 or IL6 demonstrated the advantage of cytokine co-administration with antigen in enhancing the humoral immune response (Steidler *et al.* 1998). Its poor colonizing ability has been considered the main disadvantage of using *Lactococcus lactis* as a vaccine vehicle.

1.3.2. *Streptococcus gordonii*

S. gordonii is one of the pioneer organisms in the oral cavity and likely to appear in the oral cavity as early as 6 months of age. The advantage of being a pioneer organism is that there is less competition from other organisms. The persistent nature of *S. gordonii* in the oral cavity suggests it can be a potential economical vaccine preferably to be used soon after birth (Lee *et al.* 2003). So far, there are two approaches that have been used to express heterologous proteins in *S. gordonii*. One approach is to express the protein on the surface by exploiting the C-terminal surface anchoring domain of the M6 protein or P1 antigen of *Streptococus pyogens* or *Streptococcus mutans* respectively. In the second approach, secretion of the antigen into the culture medium is made possible by using the M6 protein or P1 antigen signal sequence. Oral immunization with recombinant *S. gordonii* expressing tetanus toxin fragment C or Fim A has been shown to confer some protection against tetanus toxin lethal challenge in mice and prevent alveolar bone loss induced by *Porphyromonas gingivalis* infection in rats respectively (Medaglini *et al.* 2001, Sharma *et al.* 2001). Antigens secreted or surface expressed by *S. gordonii* are immunogenic in mucosal as well as parenteral administration. However, obtaining a high level of immune induction has been an obstacle in *S. gordonii* based vaccines.

1.3.3. *Lactobacilli*

Compared to *Lactococci* and *S. gordonii, Lactobacilli* have greater intrinsic immunogenicity and colonizing ability in the GI tract that make them potentially better candidates for vaccination. *Lactobacillus plantarum,*

Lactobacillus casei, Lactobacillus helveticus, Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus brevis and *Lactobacillus rhamnosus* GG (LGG) are commonly used *Lactobacilli* for vaccine delivery (Table 1.2). *Lactobacilli* are non invasive and the vaccine delivery to antigen presenting cells may be less effective than with invasive bacteria. Still antigen specific immune responses have been obtained with *Lactobacilli* based vaccines.

Table 1.2. *Lactobacillus* based vaccines

Bacterial strain	Antigen expressed/ secreted	Route of administration to mice	Immune response	Results	References
L. plantarum	Urease B of *Helicobactor pylori* (Intracellular expression)	Oral	specific serum antibody	partial protection against infection with *Helicobactor felis*	Corthesy *et al.* 2005
L. plantarum *L. casei*	TTFC (Intracellular expression or surface)	Oral, intranasal	Serum antibody and secretory IgA in BALF, T cell response in draining lymph nodes	provides protection against lethal challenge of tetanus toxin. Immune response elicited was *L. plantarum* > *L. casei* and intracellular TTFC > surface expressed TTFC	Grangette *et al.* 2001 Shaw *et al*. 2000
L. plantarum, *L. helveticus*	PsaA (Pneumococcal surface antigen A) antigen of *Streptococcus pneumoniae* (secretory)	Intranasal	Serum antibody and secretory IgA in BALF,	Reduction in nasal colonization of *S. pneumoniae*.	Oliveira *et al.* 2006
L. plantarum	TTFC (intracellular/surface/ secretory)	Oral, intranasal	Serum antibody and secretory IgA in BALF	Surface expression requires a lower dose to be immunogenic and high intracellular expression elicits the highest serum IgG titre.	Reveneau *et al.* 2002

L. acidophilus	*Bacillus anthracis* protective antigen (PA) fused with dendritic cell targeting peptide (secretory)	Oral	PA specific IgA, Serum IgG,neutralizing Antibody.	protective immunity against *B. anthracis.*	Mohamadzadeh, M *et al.* 2009
L. casei	Severe acute respiratory syndrome coronavirus spike protein-surface display	Oral or nasal	High serum IgG and mucosal IgA	Oral immunization renders higher neutralizing antibody production than nasal immunization.	Lee *et al.* 2006
L. casei	human papillomavirus type 16 E7 protein-surface display	Oral	Serum antibody, mucosal IgA and T cell response (ELISPOT)	Protection demonstrated against injection of E-7 expressing tumour cell line.	Poo *et al.* 2006
Cellwall mutant *L.plantarum* (alanine racemase mutants) or wild type	TTFC (intracellular)	Oral or intravaginal	High serum IgG, mucosal IgA.	In oral immunization, mutant strain was far more immunogenic than wild type. TTFC specific IgA only induced with mutant strain. Much stronger TTFC specific serum IgG was produced in intravaginal immunization of mutant strain.	Grangette *et al.* 2004

Among *Lactobacilli* based vaccines, *L. plantarum* and *L. casei* are commonly used vaccine vehicles. *L. plantarum* based TTFC vaccine delivery induced a higher TTFC specific antibody over *L.casei* in oral and intranasal immunization of C57BL/6 and Balb/c mice. Mitomycin treated *L. plantarum* induced eight fold less neutralizing tetanus antibody levels than live bacteria though it elicted almost the same level of serum TTFC specific antibody in intranasal immunization. (Grangette *et al.* 2001).

L. plantarum has also been used to induce tolerance in treating allergy. Immunization of recombinant *L. plantarum* expressing house mite allergen Der p1 or mucosal co-application of *L. plantarum* with birch pollen allergen Bet v1 could suppress the dust mite specific T cell response or pollen allergen specific Th2 allergic immune response respectively (Kruisselbrink *et al.* 2001; Repa *et al.* 2003). Recombinant *L. casei* expressing transmissible gastroentritis coronavirus spike glycoprotein or Porcine Parvovirus VP2 protein elicited antigen specific mucosal IgA or serum antibodies by intragastic administration (Ho *et al.* 2005; Xu *et al.* 2007)**.** Immunomodulatory potential of *L. casei* was demonstrated by showing significant inhibition of β-lactoglobulin specific IgE in oral immunization of BLG expressing *L. casei* (Hazebrouck *et al.* 2009).

1.3.3.1. *Lactobacillus rhamnosus* GG

Lactobacillus rhamnosus strain GG was discovered in 1985 (Gupta *et al.* 2009) and later, LGG was sub grouped as member of the *L. casei* based on cell wall peptidoglycans and the fermentation pathway of pentoses and hexoses (Hammes *et al*. 1995). LGG was the first probiotic, which received most clinical attention to date. (Gorbach *et al.* 2000). The health promoting effects

of LGG have also been studied extensively, making it the best characterized probiotic bacterium.

1.3.3.2. Benefits of using LGG

1. Survival of LGG in the human gastrointestinal tract - In human trials, LGG was shown to survive better in the gastrointestinal tract and to persist in 87% or 33% (sample size - 77) of volunteers for 4 or 7 days after oral consumption of bacteria. (Goldin *et al.* 1992)

2. Better adherence to mucosal tissue - LGG was demonstrated to have a strong

in-vitro adherence to HT-29 cells (Verdenelli *et al.* 2009). The strong adherent ability of LGG to cervico-vaginal cells and antagonistic effect on vaginosis associated pathogens also have been reported (Coudeyras *et al.* 2008).

3. Easing symptoms of gastrointestinal disorders. A clinical study done by The European society for Pediatric Gastroenterology, hepatology, and nutrition involving 287 children aged 1 - 36 months from 10 countries suffering from moderate to severe diarrhoea showed that patients receiving LGG had decreased severity and shorter duration of illness. (Guandalini *et al.* 2000). LGG was reported to block inflammatory signaling *in vivo* via reactive oxygen species generation and thereby may prevent necrotizing enterocolitis (NEC) in premature infants (Lin *et al.* 2009).

4. In alleviating allergic reactions- The immunomodulatory potential of LGG in allergic conditions has been extensively documented. LGG consumption in pregnant women for 2 - 4 weeks before their delivery date reduced the

incidence of atopic eczema in their children who were breast fed. (Kalliomaki *et al.* 2001).

5. In cancer - LGG cytoplasmic fraction exerted antiproliferative and proapoptotic effects on HGC-27 human gastric carcinoma cell lines. (Russo *et al.* 2007). LGG was also reported to induce antiproliferative or cytotoxic effects on human transitional carcinoma cell lines MGH and RT112 respectively. (Seow *et al.* 2002). LGG DNA containing novel oligodeoxynucleotide pattern elicited a strong immunostimulation in murine immune cells (Iliev *et al,* 2005). In mice bearing bladder tumours oral administration of LGG immediately after tumor cell implantation reduced the tumour size and inhibited tumour development. (Lim *et al.* 2002)

***Lactobacillus rhamnosus* GG as vaccine vehicle**

Despite of having many beneficial effects on consumption, LGG has been poorly exploited as a vaccine delivery vehicle.

1.3.4. Dose and route of administration of *lactobacilli:*

Many studies were performed to analyze the effective dose of *lactobacillus* that could induce an immune response and enhanced phagocytic cell function which has been taken as an index of immune enhancement by *lactobacillus* (Perdigon *et al.* 1986). Generally oral delivery of 10^9 cfu (LGG HN001) was found to be effective in enhancing an immune response (Gill *et al.* 2000). A dose of 10^7cfu LGG HN001 was also shown to be sufficient to enhance the phagocytic capacity of blood leukocytes but a minimum daily dose of 10^9 cfu was found necessary to enhance the phagocytic capacity of peritoneal cells

(Gill *et al.* 2001). Interestingly, there was no further increase in peritoneal cell phagocytosis by increasing the dose from 10^9 to 10^{11} cfu. However the effective dose of recombinant *lactobacilli* may vary depending on the immunogenicity and stability of the heterologous protein.

In mucosal delivery the choice of the immunization route may be crucial in inducing an effective immune response. Sometimes antibody induction may vary based on the route of immunization. For example, intranasal delivery of β-lactoglobulin (BLG) producing *L. casei* stimulated serum BLG-specific IgG2a and IgG1 responses and fecal IgA response as well, but did not inhibit BLG specific IgE induction. However intranasal immunization of recombinant lactobacilli expressing IgE mimotope induced anti IgE pecific IgG response which may be a clinical benefit for atopic patients (Scheppler et al, 2005). In contrast oral immunization of the same bacteria inhibited BLG specific IgE production while IgG2a and IgG1 responses were not stimulated (Hazebrouck *et al.* 2009). There have been controversial reports on the efficacy of the immune response with regard to the route of immunization either oral or intranasal using LAB as the vaccine (Cheun *et al.* 2004; Ramasamy *et al.* 2006; Oliveira *et al.* 2006; Robinson *et al.* 1997). Bacteria strain, booster immunization, choice of antigen expression (cytoplasmic, secreted or cell wall anchored) and scheme of immunization may determine the efficacy of immunization via different routes.

Another important factor, bacterial persistence or colonization at mucosal sites should be considered. For instance, the efficacy of intranasal immunization of lactobacilli was greater compared to *Lactococcus lactis* and the difference in immune induction was explained by low level of antigen expression in

Lactococcus lactis or the fact that lactobacilli were able to persist in mice nasal mucosa for up to 3 days while *Lactococcus lactis* were detectable only after the first day of inoculation (Oliveira *et al.* 2006).

1.3.5. Immunomodulatory functions of *lactobacilli* on dendritic cells and neutrophils:

Dendritic cells (DC) play a pivotal immunoregulatory role in the Th1, Th2, and Th3 cell balance and are present throughout the gastrointestinal tract. Thus, DC may be targets for modulation by gut microbes, including ingested probiotics. The different species of *Lactobacillus* differentially activate DC. When bone marrow-derived murine DC were exposed to various lethally irradiated *Lactobacillus* spp, almost all different strains up-regulated surface MHC class II and B7-2 (CD86), though they induced diffrential cytokine production from DC. Significant differences among the lactobacilli species were observed for the production of IL-12 and TNF-α with the following ranking of the species *L. casei* >> *L. plantarum* Lb1 > *L. fermentum* ~*L. johnsonii* ~*L. plantarum* >> *L. reuteri* (Christensen *et al*, 2002). Lactobacilli activated human dendritic cells skew T cells toward T helper 1 polarization. Lactobacilli treated monocyte derived human dendritic cells co-cultured with T cells induced allogeneic or autologous $CD4^+$ and $CD8^+$ T cell proliferation (Mohamadzadeh *et al*, 2005) .

Recently more interest has been shown on the role of neutrophils in antigen presentation. Neutrophils may influence T cell responses to bacteria, either by directly presenting peptide-MHC-I complexes or by delivering peptides to other APCs for presentation (Potter *et al*, 2001). Neutrophils were recently

shown to be able to cross present antigens to cytotoxic T cells. Cross-presentation by neutrophils was TAP and proteasome dependent and was as efficient as in macrophages. Moreover, it actually occurred earlier than in professional antigen-presenting cells.

However only limited literature is available on lactobacilli mediated direct immunomodulatory effect on neutrophils. *In vitro* experiments with *Lactobacillus plantarum* showed an inhibited intestinal epithelial migration of neutrophils induced by enteropathogenic Escherichia coli (Michail *et al*, 2003). Primary culture of peritoneal neutrophils treated with *L. casei* lysates showed higher Nitric Oxide production and demonstrated an enhanced phagocytotic and free radical scavenging activity (Lee *et al*, 2010). *Lactobacillus casei* treatment restored neutrophil phagocytic capacity in cirrhosis, possibly by changing IL10 secretion and TLR4 expression (Stadlbauer *et al*, 2008). However lactobacilli mediated indirect DC activation by neutrophils has not been evaluated.

1.4. Role of promoter and cellular location (surface/intracellular/cell wall anchoring/ secretory) of antigen in immune induction

Studies of gene expression and regulation in *lactobacilli* have received more attention recently for their potential role in heterologous expression of protein especially industrially important enzymes. Constitutive and inducible promoter systems in *lactobacilli* have been well exploited to express foreign proteins. A constitutive promoter system is a natural choice for colonizing *lactobacilli* during *in situ* production in the human body or when steady state gene expression is required (Jensen *et al.* 1993). Efficient and established

constitutive promoters used in *lactobacilli* were lactate hydrogenase (ldh) and surface layer protein A (slpA) promoters (Table 1.3). In constitutive expression, continuous high-level of production of a protein could lead to intracellular accumulation, aggregation, or degradation of protein in the cytoplasm which is deleterious to cells (Makrides *et al.* 1996). In that case, inducible expression would be the best choice. Inducible expression may also be preferable in cases where the aim is to overproduce a desired protein at high levels. For inducible promoters, several expression systems have been constructed for *Lactococcus lactis* (Sorvig *et al.* 2003, 2005). Among the inducible promoters, the bacteriocin inducible system is one of the most commonly used (Table 1.4). Many LAB produce antimicrobial peptides called bacteriocins and their production is regulated by the secreted peptide pheromone which activates a two component regulatory system consisting of a histidine kinase receptor and cognate response regulator. In strains, producing class I bacteriocins (such as nisin), the bacteriocin itself acts as pheromone (Kuipers *et al.* 1995). Strains producing class II bacteriocins, such as sakacin A and sakacin P produce a separate peptide pheromone (Nes *et al*.1999). In both cases, pheromones activate the transcription of all the operons involved in bacteriocin production through the response regulator. Though the best characterized and most commonly used controllable expression system is the nisin controlled expression (NICE) system, in which nisin serves as an inducer, (de Ruyter *et al.* 1996a; de Ruyter *et al.* 1996b) it often exhibits significant basal activity, i.e. activity without activation. (Eichenbaum *et al.* 1998; Pavan *et al.* 2000). In that case inducible expression system involving class II bacteriocins may be preferred.

Table 1.3. Heterologous protein expression under constitutive promoters in LAB

Promoter	Source	Host for expression	Cellular location of the antigen	Level of protein expression	References
Constitutive					
cbh (conjugated bile salts hydrolase)	*L. plantarum*	*L. planatarum*	Intracellular	2% of the total protein	Pouwels *et al.* 1996
ldh (lactate dehydrogenase)	*L. casei*	*L. plantarum*	Intracellular	1-2% of the total protein	Pouwels *et al.* 1996
ldhUTLS (untranslated leader sequence)	*L. casei* (core promoter) and *L. acidophilus* (untranslated leader sequence)	*L. casei*	Surface display using anchor protein *Bacillus subtilis* subsp. *chungkookjang* PgsA	Not determined	Narita *et al.* 2006
ldh	*L. casei*	*L. casei* Shirota	Secretory- using secretion signal of *prt* P gene of *L. casei.*	Not determined	Ho *et al.* 2005
slpA (S-layer protein)	*Lactobacillus brevis*	*Lactococcus lactis, L. brevis, L. plantarum*	Secretory- using *L. brevis* S layer protein A secretory signal	50 µg/ml in *Lactococcus lactis*, 30 µg/ml in *L.brevis* and 15 µg/ml in *L. plantarum*	Savijoki *et al.* 1997
Pgm (Phosphoglyceromutase)	*L.acidophilus*	*L. acidophilus*	Secretory	not determined	Mohamadzadeh *et al.* 2009

Table 1.4. Heterologous protein expression under Inducible promoter in LAB

Promoter	**Source**	**Host for expression**	**Cellular location of antigen**	**Level of protein expression**	**References**
Inducible					
P_{nisA} (Nisin inducible)	*Lactococcus lactis*	*Lactococcus lactis*	cytoplasmic, Secretory and cell wall anchored	0.5 µg/ml 3 µg/ml not determined.	Ribeiro *et al.* 2002
P_{nisA} (Nisin inducible)	*Lactococcus lactis*	*Lactococcus lactis*	surface anchored	not determined	Bermudez-Humaran *et al.* 2004
P_{nisA} (Nisin inducible)	*Lactococcus lactis*	*Lactococcus lactis*	secretion of murine IL12p35-p40 heterodimer using signal peptide of usp45 (SP_{usp45})	25 pg/ml	Bermudez-Humaran *et al.* 2003
P_{sapA}, P_{sapIP}, P_{orf1} (in pSIP300 series)-Sakacin A or P_{sppA}, P_{orf330}, P_{orfx} (pSIP400 series) -Sakacin P inducible)	*Lactococcus lactis* *L. plantarum*	*Lactococcus lactis* *Lactobacilli*	measured GusA activity	induction factor was about 127 in *L. sakei* using pSIP409 plasmid compared to 24 in *L. plantarum* using two plasmid NICE system.	Sorvig *et al.* 2005
P_{orfx} (Sakacin P inducible)	*L. plantarum*	*L. plantarum*	cytplasmic expression of oxalate decarboxylase from *Bacillus subtilis*	enzymatic activity was about 19.8 U/mg which is same as the yield obtained with *E. coli* expression system.	Kolandaswamy *et al.* 2009

1.5. Mucosal vaccine- challenges

In recent years, the use of recombinant LAB for mucosal delivery has been given more attention. Despite much progress, there are several issues that still need to be addressed.

1. LAB vaccines that are administered orally or intranasally get diluted in mucosal secretion and trapped in mucus gel. Relatively larger doses of vaccine may be required and it is hard to determine what dose actually crosses the mucosal layer.

2. The most frequently asked question about the LAB based mucosal vaccine is the possible induction of mucosal tolerance. Repeated oral ingestion of antigen results in decreased or totally abrogated responsiveness to subsequent systemic immunization with the same antigen. Though some LAB are intrinsically immunogenic, additional adjuvants like cholera toxin (CT) or cytokine co-expression may help to avoid tolerance induction.

3. Recombinant bacterial vaccines when administered induce immune responses against the vaccine antigen initially, but the response to the neo-antigen is overwhelmed by response to the more immunodominant antigen of the bacteria itself.

4. The effectiveness of the live bacterial vaccines is partly dependant on the transport to organised lymphoid tissues. Vaccines derived from pathogenic microbes like live attenuated *S. typhi* (Levine *et al.* 2000) or live attenuated polio virus (Modlin *et al.* 2004) preferentially adhere to M cells and exploit M-cell transport to invade organized mucosal lymphoid tissues in the intestines (Jones *et al.* 1994; Sicinski *et al.* 1990). *Lactobacilli* are not known to invade lymphoid

tissue as the microbes discussed above. However expressing foreign protein with DC targeting peptide may improve bacterial uptake (Mohamadzadeh *et al.* 2009).

1.6. Scope of study

To date, there is limited literature documenting the use of LGG as a vaccine vehicle. Our hypothesis is

LGG can be a good antigen delivery vehicle in mucosal immunization and this dissertation aims to characterize LGG as vaccine delivery vehicle in the following manner.

Objectives of the work

a) Study the humoral and cellular immune response by oral or nasal immunization in mice using GFP (Green Fluorescent Protein) as a model antigen expressed in LGG.

b) Study the advantage of Interleukin-2 (IL2) co-expression with antigen in enhancing specific immune induction

c) Study the dendritic cells and neutrophils mediated antigen presentation of IL15 or IL2 or IL7- Prostate Specific Antigen (PSA) fusion protein secreting LGG to activate T cells.

d) Study the compatibility of different promoters in LGG and optimization of the promoter for efficient secretion of the antigen.

Chapter Two
Materials
and
methods

2. Materials and methods

2.1 Production of *Lactobacillus rhamnosus* GG expressing protein antigens

2.1.1 *Lactobacillus rhamnosus* strain GG (LGG)

Lactobacillus rhamnosus GG (ATCC 53013) (a kind gift from Dr Seppo Salminen of University of Turku, Finland) growth curves were produced by plotting OD550 nm versus number of bacterial colonies of freshly prepared, serially-diluted cultures grown on de Man, Rogosa, Sharpe (MRS) (Merck, Darmstadt, Germany) agar plates. Bacteria were harvested at the late log phase (OD550 nm for L. rhamnosus was 5.2)

and the CFU were approximately 3 x 10^9 colony forming units/ml (cfu/ml). Overnight cultures of LGG were routinely used for animal experiments. The bacteria were grown and maintained in MRS media (Merck) at 37^{o}C. To avoid strain variation from prolonged culture, new LGG glycerol stocks were thawed to start a fresh culture every 2 - 3 months.

2.1.2. Plasmid for protein expression in *Lactobacillus.*

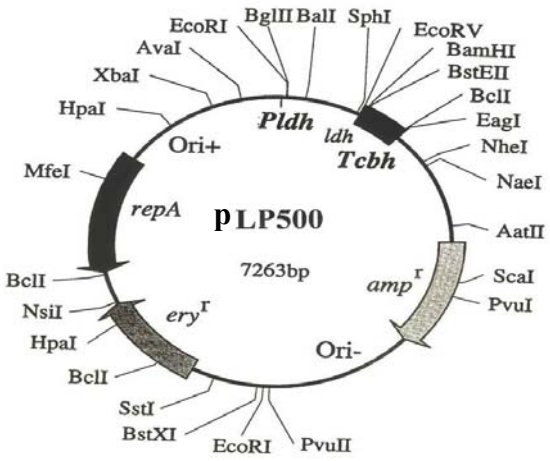


Figure 2.1. Restriction map of *E. coli- Lactobacillus* shuttle vector, pLP500

The *Lactobacillus* - *E. coli* shuttle vector pLP500 (Fig 2.1), was obtained from Prof. Pouwels PH of the TNO-Nutrition and Food Research Institute, The Netherlands. The plasmid contains the constitutive promoter of the L-(+)-lactate dehydrogenase

(L-ldh) gene, downstream of the secretory signal of the *prt* P gene of *L. casei* which confers secretable expression (Ho *et al.* 2005) It also carries both ampicillin and erythromycin resistance genes for selection in *E.coli* or *Lactobacilli* respectively.

2.1.3. LGG-green fluorescent protein (LGG-GFP)

LGG-eGFP (enhanced Green Fluorescent Protein) was a gift from Prof. Chua Kaw Yan, Dept of Pediatrics, National University of Singapore, Singapore. LGG-GFP was cultured in MRS media supplemented with 10 μg/ml erythromycin (Sigma-Aldrich, St. Louis, MO)

2.1.4. Cloning of murine Interleukin-2 (IL2) gene to generate IL2-GFP fusion protein

The mature peptide sequence (nucleotides 108 - 554bp) encoding mouse interleukin-2 was amplified from pBUD-IL2 plasmid by polymerase chain reaction using the primers listed in Table. 2.1(a). The 3' primer was designed without its termination codon and after subcloning into the pLP500 vector at the N terminus of the GFP gene, 2 amino acids, glycine and serine were inserted before

the GFP protein sequence. The construct pLP500-IL2-GFP was verified by nucleotide sequencing.

2.1.5. Genomic DNA extraction from *L. acidophilus*

Genomic DNA from *L. acidophilus* was extracted as described before (Martin-Platero *et al.* 2007) with some modifications. An overnight culture (50-100ml) of *L. acidophilus* was centrifuged and resuspended in 100 ml TES buffer (10% sucrose, 25 mM Tris HCl pH 8.0, 10 mM EDTA). Then 10 mg/ml of freshly prepared lysozyme, 100 U/ml mutanolysin and 40 µg/ml of RNase were added and incubated for 30 minutes at 37°C. Cells were pelleted down and lysed with 600 µl of lysis buffer (100 mM Tris HCl pH8.0, 100 mM EDTA, 10 mM NaCl and 1% SDS) by mixing gently and incubated for 10 to 15 minutes at room temperature. The lysate was treated with proteinase K (10 mg/ml) and incubated for 15 minutes at 37°C followed by another incubation at 80°C for 5 minutes. Then samples were allowed to cool to room temperature for 10 minutes. 200 µl of 3M sodium acetate pH 5.2 was added and the sample was vortexed for 10 - 15 seconds and chilled for 10 - 15 minutes. Then the sample was centrifuged at 20,000x g for 10 minutes to precipitate proteins. The supernatant was transferred to a new tube and DNA was precipitated by adding an equal volume of isopropanol. The tube was inverted several times and DNA was precipitated by centrifugation at 20,000x g for 5 minutes. The pellet was washed once with 1 ml 70% ethanol and subsequently dried at room temperature. Finally the DNA was resuspended in 200 µl of TE buffer and analyzed by gel electrophoresis.

2.1.6. Replacement of the *ldh* promoter of pLP500 with the *slpA* promoter to produce pLP500-slpA$_P$ plasmid

The *slpA* (S-layer protein gene) promoter with secretory signal peptide sequence was amplified by PCR using the primer pair listed in Table 2.1(a) which were designed to have a *Bgl*II site at the 5'end and *EcoR*V site at the 3'end respectively. This amplified PCR product replaced the P*ldh* promoter in pLP500 by restriction digestion with *Bgl*II and *EcoR*V and the plasmid produced was named pLP500-slpAp.

2.1.7. Producing different promoter constructs to modify antigen secretion

The core *ldh* promoter without ribosome binding site (RBS) or coding sequence was amplified by PCR from genomic DNA of *L. casei* using the primer pair listed in Table 2.1 (b) which were designed to have a *Bgl*II site at both 5' and 3'ends and subcloned in to pLP500-slpAp plasmid, upstream to *slpA* promoter sequence. The construction and orientation of the P*ldh* insert was confirmed by sequencing.

The putative promoter of *L. acidophilus* pgm gene was amplified from genomic DNA by PCR using the primer pair listed in Table 2.1(b) which were designed to have a *Bgl* II site at the 5' end and *EcoR*V at 3' end and subcloned into *Bgl*II, *EcoR*V digested pLP500 plasmid to produce pLP500-pgmp plasmid. The construction was confirmed by sequencing.

2.1.8. Cloning of murine IL2 in pLP500ldh-slpAp (tandem promoter) or pLP500-pgm$_P$ plasmid

Mouse interleukin-2 was amplified from pBUD-IL2 plasmid as described above and subcloned in to pLP500ldh-slpAp or pLP500-pgmp and or pLP500. The constructs were verified by nucleotide sequencing.

2.1.9. Cloning of human Prostate Specific Antigen (PSA) or murine IL2 or IL15 or IL7 in pLP500-slpA$_P$ plasmid.

A 0.69 kbp cDNA fragment (nucleotides 136 - 827), encoding PSA or kallikrein-related peptidase 3 (KLK3) gene was amplified from pSec Tag2/Hygro/PSA plasmid (Invitrogen, CA, USA) by PCR using the primers listed in table 2.1(a) and subcloned into pLP500 plasmid, downstream to the *slpA* promoter sequence. The construction of the gene and whether the psa gene was in frame with *slpA* signal peptide sequence were confirmed by sequencing. For mouse IL2, IL15 and IL7 the primers listed in Table 2.1(a) were used to amplify the mature pepetide sequence of the respective cytokines which were cloned singly and upstream of PSA.

Table 2.1 (a). Plasmids generated

Plasmid/ DNA Source	Primers	Plasmid produced/ recombinant LGG
Genomic DNA of *L. acidophilus*	Nucleotide -286 to + 111 of the slp promoter region with secretion signal forward primer 5' GGG AGA TCT TGC TTG TGG GGT AAG CGG TAG 3' and Reverse primer 5' GAT ATC TGC GTT AAT AGT AGT AGC AGC GC '3 which contained *Bgl* II and *Eco*RV sites	pLP500slpAp / LGG-S
pSec Tag2/Hygro/PSA	nucleotide 136-827 of PSA gene (codes for mature protein minus 8aa) forward primer 5' CC GAT ATC ATG GAG AAG CAT TCC CAA CCC 3' and Reverse primers 5'GGG GAT CC TCA GGG GTT GGC CAC GAT GGT 3' which contained *Eco*RV and *BamH*1	pLP500slpAp PSA/ LGG-S-PSA
pBud- IL2	mature peptide nucleotide sequence 108-554bp Forward primer 5' GGG GAT CC GCA CCC ACT TCA AGC TCC AC 3' and Reverse primer 5'GAT GGG GAT CC TTG AGG GCT TGT TGA GAT which contained BamH1 at both sites mature peptide nucleotide sequence 108-554bp Forward primer 5'CC GAT ATC GCA CCC ACT TCA AGC TCC AC 3' and Reverse primer 5'GAT GGG GAT CC TTG AGG GCT TGT TGA GAT 3' which contained *Eco*RV and *BamH*1	pLP500ldhp IL2- GFP LGG-IL2-GFP pLP500slpAp IL2- PSA/ LGG-S-IL2-PSA
	Reverse primer 5' GAT GGG GAT CC T TAT TGA GGG CTT GTT GA3'	pLP500slpAp IL2/ LGG-S-IL2
pCMV-SPORT6 IL15 (ATCC)	Mature peptide encoded by nucleotides 610-951bp Forward primer 5'CC GAT ATC AAC TGG ATA GAT GTA AGA TAT G 3' and Reverse primer 5' GGG GAT CC GGA CGT GTT GAT GAA CAT 3' which contained *Eco*RV and *BamH*1	pLP500slpAp IL15-PSA/ LGG-S-IL15-PSA
	Reverse primer 5' GGG GAT CCT CAG GAC GTG TTG ATG AAC AT3'	pLP500slpAp IL15/ LGG-S-IL15
Mouse bone marrow cells	Mouse IL7 mRNA encoded by nucleotides 247-712 bp Forward primer 5' GG GAT ATC ATG TTC CAT GTT TCT TTT AGA 3' and Reverse primer 5' GGG GGA TCC TAT ACT GCC CTT CAA AAT TTT 3' which contained *Eco*RV and *BamH*1	pLP500slpAp IL7-PSA/ LGG-S-IL7-PSA
	Reverse primer 5' GGG GGA TCC TTA TAT ACT GCC CTT CAA AAT TTT	pLP500slpAp IL7/ LGG-S- IL7

Table 2.1 (b). Plasmids generated

Plasmid/DNA source	Primers	Plasmid produced/ recombinant LGG
pLP500slpAp/ Genomic DNA of *L. caesei*	Nucleotide -511 to -18 of the ldh promoter region Forward primer 5' GGG AGA TCT GAA TTC AGA TCT ACT AGA GGA TCT GTG 3' Reverse primer 5' CCC AGA TCT TTA TGT GCA TGC AAA CTG C 3'. which contained *Bgl* II at both sites.	pLP500 ldh-slpAp/ LGG-LS
pLP500/ Genomic DNA of *L. acidophilus*	putative promoter region of pgm gene. Forward primer 5' GGG AGA TCT TGC GAC AAG TAA TAA ACT AAA C 3' Reverse primer 5' CCC GAT ATC AGC CTT CTT AGC TTC TTC AAC A 3' which contained *Bgl* II and *EcoR*V sites.	pLP500pgmp LGG-P

2.1.10. Preparation of LGG electrocompetent cells

Preparation of electrocompetent LGG was done as described before (De Keersmaecker *et al.* 2006) with some modifications. An overnight culture of LGG was inoculated into prewarmed MRS medium supplemented with 2% glycine and was incubated without agitation at 37°C. After overnight growth, 5ml of the culture in the exponential growth phase (OD 600 0.8 to 1) was inoculated into 100 ml of prewarmed MRS medium supplemented with 2% glycine. The resulting culture was kept in a tightly closed 100ml flask and incubated without agitation at 37°C. When the OD 600 was 0.2 to 0.3, ampicillin (10 μg/ml) was added and bacteria were further incubated to an OD 600 reading of 0.4 to 0.5. Cells were harvested by centrifugation at room temperature for 10 minutes at 4000x g. The cells were washed three times with electroporation buffer (0.4 M sucrose, 5 mM potassium dihydrogen phosphate [pH 6.0], 1 mM $MgCl_2$) at room temperature and finally resuspended in 1 ml of the same buffer and placed on ice. The

electrocompetent cells were used immediately for electroporation.

2.1.11. Electroporation of LGG.

Electroporation was performed as described before (De Keersmaecker *et al.* 2006). A mixture containing 100 µl of a cooled cell suspension and 400 ng of plasmid DNA

(maximum volume 5 µl) was transferred into a precooled electroporation cuvette (Bio-rad, CA, USA) with a 0.2 cm electrode gap and immediately electroporated (Gene Pulser: Bio-rad) using the following settings; peak voltage, 1.7 kV:; capacitance, 25 µF: and parallel resistance, 200Ω. Following the pulse, the cells were immediately diluted with 5 ml of MRS medium containing 2mM $CaCl_2$ and 20mM $MgCl_2$ and incubated for 37°C for 3 hours without agitation before they were plated onto MRS agar plates containing the 10 µg/ml of erythromycin. Plates were incubated at 37° C, 5% CO_2 for 48 to 72 hours.

2.1.12. Determination of IL-2 or IL-15 biological activity

The IL2 dependent mouse cytotoxic T lymphocyte cell line – CTLL-2 (ATCC, Manassas,VA,USA) (ATCC number- TIB-214™) was purchased from ATCC and maintained in RPMI 1640 media (Sigma Aldrich) supplemented with 2 mM L-glutamine (Sigma Aldrich), 1 mM sodium pyruvate (GIBCO BRL, CA, USA), 10% v/v foetal bovine serum (FBS) (Hyclone, USA) and 5000U/ml Penicillin and 5 mg/ml Streptomycin (Sigma Aldrich) and 10% T-STIM with Con A (BD Biosciences, San Jose, CA, USA) . For the proliferation assay, the cells were

washed twice and suspended at 1.4 x 10^5 cells per ml in IL-2 free media (T-STIM with Con A) as described by Vorauer *et al.* 1996. Recombinant human IL-2 (e-bioscience, San Diego, CA,USA.) from 20 to 0.3 U/ml or conditioned media, that was prepared from bacterial supernatant (from LGG-IL2 [ldh_p] or LGG-IL2-GFP or LGG-IL2[$SlpA_P$] or LGG-IL15 or LGG-IL2-PSA or LGG-IL15-PSA) dialyzed against blank RPMI media were added to the cells for 28 - 72 hours. Cell proliferation was determined by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA).

2.1.13. Analysis of cytokines, PSA or GFP expression

GFP expression in the bacteria was analyzed on a Coulter EPICS ELITE ESP flow cytometer. GFP in LGG-GFP or LGG-IL2-GFP culture supernatant or bacteria lysates were determined by ELISA (Reacti-Bind Anti GFP coated plates, Pierce biotechnology Inc, Rockford, IL, USA) and GFP fluorescence was detected by a luminescence spectrometer (Perkin-Elmer LS50B, Waltham, Massachusetts, USA) (De Keersmaecker *et al.* 2006).

Recombinant LGG were initially inoculated in 5ml of MRS broth with 10 µg/ml of erythromycin (Sigma Aldrich) and incubated at 37ºC for 8 hours. Then the culture was diluted 20 times with MRS broth containing 10 µg/ml of erythromycin and incubated up to 20 - 22 hours when the culture supernatants were harvested and analyzed for cytokine or PSA. IL2 (BD Biosciences), IL15 (eBiosciences) and IL7 (Ray biotech, Norcross GA, USA) were analyzed with commercial ELISA kits. For ELISA, briefly, 100 µl capture antibody (Ab) diluted in coating buffer was

added to each well, and incubated overnight (4°C) except for IL7 in which precoated plates were used. The plates were then washed three times with wash buffer (PBS with 0.05% v/v Tween 20) and blocked with 200 µl assay diluent (RT for 1 hour). Following which, the plates were washed 3 times before the addition of 100 µl standard or bacterial supernatant from LGG (neat or diluted). Plates were incubated for 2 hour at RT. The plates were washed again, then 100 µl working detector (biotinylated detection Ab and streptavidin-horseradish peroxidase conjugate [HRP]) was added to each well (RT, 1hour). The plates were then washed before 100 µl TMB substrate solution (Pierce Biotechnology Inc) was added to each well, and incubated in the dark (RT, 30 min). The reaction was stopped with the addition of 50 µl stop solution (2N H_2SO_4) to each well. The absorbance was read at 450 nm; reference wavelength 570 nm (Tecan GENios/ Magellan, Männedorf, Switzerland). For PSA ELISA, MaxiSorp™ immuno modules (NUNC, Roskilde, Denmark) were coated overnight at 4°C with 1 µg/ml rabbit anti human PSA antibody (US Biologicals, Swampscott, MA) in 1x Tris Buffered Saline (TBS – 50 mM Tris, 150 mM NaCl, pH adjusted with HCl to 7.4). Then the plates were washed once with washing buffer (TBS containing 0.1% Tween 20) and blocked with 300 µL of TBS containing 2% BSA for 3 hours at RT with gentle shaking. Subsequently the plates were washed 3 times before either 50 µL of PSA standards (Chemicon, Temecula, CA) and dialyzed bacterial supernatant (neat or diluted in TBS containing 0.05% BSA) were incubated for 1 hour at RT with gentle shaking. The plates were washed 5 times with the washing buffer before adding 100 µL of the secondary antibody (1 µg/mL mouse anti-PSA

monoclonal antibody conjugated with horseradish peroxidase, US Biologicals, Swampscott, MA) diluted in 0.5% BSA and 0.1% Tween 20 in TBS. After 1 hour at RT with gentle shaking, the plates were washed 5 times with the washing buffer. The plate was then developed using 1-Step™ Turbo TMB-ELISA substrate (Pierce Chemical Co.) for 30 minutes at RT in the dark. The reaction was stopped by adding 2N H_2SO_4 and the absorbance was read at 450 nm; reference wavelength 570 nm. The Cytokine or PSA concentration read off the appropriate standard curve. Cytokines or PSA secretion were expressed as ng/ml of supernatant.

2.2 In vivo analysis of LGG vaccines

2.2.1. Animals

4 – 6 week old female C57BL/6 mice were maintained at the National University of Singapore's (NUS) Animal Holding Unit throughout the duration of the experiment with food and water *ad libitum*. The mice were acclimatized for a week prior to the start of each experiment. All experiments were performed according to guidelines set by the NUS Institutional Animal Care and Use Committee (IACUC).

2.2.2. Translocation of bacteria

Four groups (n=12/group) of mice were immunized via the nasal or oral route with "live" 10^8 LGG-GFP or LGG-IL2-GFP, or with sterile PBS. Four mice from each group were euthanized after 24h, 48h and 1 week. NALT, cervical lymph node

(CLN) and mediastinal lymph Nodes (Med.LN) or Mesentric lymphnodes were harvested in intranasally or orally immunized mice respectively. Spleen and liver tissues were also collected and the tissues were homogenized in an ice bath using polytron homogenizer equipped with metal probe. The homogenates were serially diluted with sterile saline and plated on MRS agar plates containing 10 µg/ml erythromycin. Bacterial colonies were enumerated after 48 – 72 hours and data was expressed as CFU per gm of tissue.

2.2.3. Intranasal immunization protocol and immune cells, cytokine analysis in BAL (Bronchoalveolar lavage) fluid

For intranasal immunization, four groups with twenty mice each were immunized under anesthesia with either 10^8 LGG or LGG-GFP or LGG-IL2-GFP or with PBS (20 µl) on days 0, 14 and 28 as previously described (Steidler *et al.* 1998). Four mice from each group were euthanized on days 0, 10, 24, 35 and 80 and the serum and BAL fluid were collected (Hopfenspirger *et al.* 2002) and centrifuged to separate the cells from the fluid. The BAL supernatant was stored in aliquots at -80ºC for cytokine analysis. The cells were H & E stained and enumerated.

NALT and CLN were harvested on the 10^{th}, 24^{th} and 35^{th} days and single cell suspensions were prepared for flow cytometry. Collected NALT tissues were mashed and single cell suspension was prepared by filtering through a 70 µm cell strainer (BD Falcon, USA). CLN tissues were placed in a 6-well culture plate on ice and cut into fine pieces. Subsequently they were digested with collagenase (Sigma Aldrich) resuspended in complete media (1 mg/ml per tissue) for 30min at

37^{o}C with shaking. The suspension was then filtered through a 70 μm cell strainer (BD Falcon, USA) and collected in a 2 ml tube, then centrifuged at 8,000 rpm (4^{o}C, 2 min). The supernatant was removed and the pellet subjected to red blood cell (RBC) lysis using RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). The lysed suspension was then centrifuged at 8,000 rpm (4^{o}C, 2 min) (Eppendorf Centrifuge, Hamburg, Germany) and the cell pellet was rinsed twice with cold PBS. The cells were finally resuspended 1 ml PBS with 0.01% sodium azide, 1% (w/v) bovine serum albumin (BSA) and 0.1% formaldehyde for flow cytometry analysis. Single cell suspensions from the CLN were stained with antibodies to B220, IgA, CD4, CD8, CD3, Mac-3 (M3/84) and pan NK(DX5) (BD Pharmingen, USA) while those from the NALT were stained with B-220 and IgA antibodies. About 1.0 x 10^4 cells were examined on a Coulter flow cytometer (Fullerton, CA, USA) and analyzed with Win MDI software.

Table 2.2. Primer sequences for Reverse transcriptase polymerase chain reaction (RT-PCR)

Gene	Primer sequence 5'-3'	Annealing temperature	Expected PCR product size
Rps27a F	ACCATCACGCTCGAGGTTGA	55	333
Rps27a R	AGGACACTCTCGACGAAGTC		
CCL2 F	GCATCCACGTGTTGGCTCAG	60	383
CCL2 R	CACACTGGTCACTCCTACAG		
CCL6 F	ATCCTTGTGGCTGTCCTTGG		
CCL6 R	GGCACCTCTGAACTCTCC	60	268
CCL17 F	GGTCACTTCAGATGCTGCTC		
CCL17 R	TCTGCACAGATGAGATTGCC	60	220
CCL25 F	AGGTGCCTTTGAAGACTGCT		
CCL25 F	TCACCATCCTGGGATGACCT	53	337
CCR2 F	GAGCCTGATCCTGCCTCTAC		
CCR2 R	GGCACTGTTTGAAGAGACGT	58	371
CXCL5 F	GACTCTGACCCCAGTGAAG		
CXCL5 R	GTGAGATGAGCAGGAAGC	53	125
FCγ r1 F	ACCTCCCTCAGGTCCAGATA		
FCγ r1 R	GTGACACCCATGTAAGCCGT	57	264
IL1r1 F	CTCATGGTGCCTCTGCTGTC		
IL1r1 R	CCCTCTGTGCTCTTCAGCCA	60	529
IL16 F	GACCCAAGGCAAGCTGTGAT		
IL16 F	CTGGAGGCTGGTCCTTCTGA	58	399
IL10 F	GAGACTTGCTCTTGCACTAC	53	634
IL10 R	CCTGGAGTCCAGCAGACTCA		
IL20 F	ATGAAAGGCTTTGGTCTTGC	51	527
IL20 R	TAGCATCTCCTCCATCCATCT		
IL2rg F	CTTCCAGAGGTTCAGTGCTT	53	489
IL2rg R	CTCCGAACCCGAAATGTGTA		
IL5ra F	CTGCCTTTCCAGATCATTGC	53	436
IL5ra R	GGGCCGGAACCGGTGGAAA		
IP10 F	ATGAACCCAAGTGCTGCCGTC	57	358
IP10 R	TGGAGAGACAGGCTCTCTGCT		
CD8a F	AAATGGACGCCGAACTTGGT	60	272
CD8a R	CTGAGCAGAAATAGTAGCCTTCG		

2.2.4. Expression of inflammatory cytokines and receptors in mice lung after 35th or 80th day of post primary intranasal immunization

Mice lungs were harvested on the 35th or 80th day of post primary nasal immunization and one lung from each treatment group was used to probe a mouse inflammatory cytokines and receptors Oligo GEArray (SuperArray, USA) according to manufacturer's instructions. Briefly, total RNA was extracted using Trizol (Invitrogen Life Technologies, Inc.) and poly A^+ RNA was isolated using Oligotex mRNA Kit (Qiagen, Germany). Biotinylated cRNA was synthesised from the purified poly A^+ RNA using the TrueLabeling-AMP Linear RNA Amplication Kit (SuperArray, USA). The target cRNA probe (5 μg) was allowed to hybridize to the membrane for 24 hours. The chemiluminescent array image was recorded on X-ray film and the scanned TIFF image of the array was then analyzed with the GEArray Expression Analysis Suite.

2.2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

To corroborate the oligo array data, lungs from immunizaed mice on 35th or 80th day of post primary immunization were harvested. Total RNA (1 μg) was used to generate cDNAs with 80 units M-MuLV Reverse Transcriptase (New England Biolabs, USA) with specific primers sets (Table 2.2) for amplification of GAPDH, chemokine (C-C motif) ligand 2, Ccl6, Ccl17 and Ccl25 Chemokine (C-X-C motif) ligand 5, chemokine (C-C motif) receptor 2 and Fc gamma receptor 1 (Fcγ r1).

For IL1rl,, IL10, IL16, IL2rg, IL5ra and IL20, 5 μg total RNA and 80 units of Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen Life Technologies, Inc.) were used. Specific primer sequences are listed in Table 2.2.

The PCR assay conditions for the housekeeping gene, Rps27a were: 94°C for 5 min, 30 cycles of amplification (94°C for 45 sec, 55°C for 45 sec [annealing temperature], 72°C for 60 sec), and an additional extension step of 72°C for 5 min. Primer sequences for all other genes are also listed in Table 2.2 along with their respective annealing temperature. PCR products were separated on a 1.5% agarose gel containing ethidium bromide and then photographed under ultraviolet light. The message band intensities were quantified with SynGene GeneTools analysis software. The values of the mRNA transcripts were normalized against Rps27a.

2.2.6. Histopathological analysis of the immunized mice lungs

Lung tissues were harvested on the 35th and 80th days, fixed with 10% phosphate-buffered formalin and embedded in paraffin for sectioning. Sections 4μm thick were cut and stained with hematoxylin and eosin (H & E) reagent to evaluate the general morphology. The specimens were examined by Dr. Nilesh Shah, pathologist, Dept of Pathology, NUS who was blinded to the treatment groups.

2.2.7. Immunohistochemical staining

Lung tissues were harvested on the 80th day and snap frozen in isopentane (Sigma Aldrich) prechilled in liquid nitrogen and stored at -80ºC. 10 μm Cryosections (Leica microsystem GmbH) were air-dried on poly-L-lysine coated glass slides.

They were overlaid and incubated for 1 hour at RT with 5% normal goat serum (Dako).

B cells or IgA were stained with FITC conjugated anti-mouse CD45R/B220 (Biolegend) or rabbit anti mouse IgA (Zymed, Invitrogen Life Technologies, Inc) respectively for 1 hour following manufacturers instructions. After washing with PBS (3 x 5min) each sections were treated with rhodamine conjugated goat anti rabbit IgG (US biologicals) for 1 hour. Cells were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics) according to the manufacturer's instructions. The slides were mounted using mounting medium and visualized under fluorescence microscope (Carl Zeiss, Inc., Thornwood, USA).

2.2.8. Oral immunization protocol and immune cell analysis in mesenteric lymph nodes (MLN)

Four groups with eight C57BL/6 or Balb/c mice in each were immunized by oral gavage with either 10^8 wild type LGG, LGG-GFP, LGG-IL2-GFP or with PBS on days 0, 14 and 28 (Steidler *et al*. 1998). Sera and feces were collected on days 0, 10, 24, 35 and 80. In a second set of experiments, on the 35th day the MLN from C57BL/6 mice were harvested and single cell suspensions were prepared for staining with antibodies to B220, IgA, CD4, CD8, CD3 (BD Pharmingen, USA) for flow cytometric analysis. About 1.0 x 10^4 cells were examined on a $CyAn_{ADP}$ (Dako Cytomation, Sweden). Data was analyzed with Summit software (Dako Cytomation).

2.2.9. Intestinal fragment cultures from orally immunized mice.

The Peyer's patches were removed from the harvested small intestine and transverse colon of mice, washed with PBS to remove the fecal content and then 100mg of tissue were incubated in 1ml of RPMI supplemented with 10 % FBS; 100 U/ml penicillin; 100 µg/ml streptomycin; 0.25 µg Amphotericin B (GIBCO); 2 mM L-glutamine; 1 mM sodium pyruvate (GIBCO); 5 x 10^{-5} M 2-mercaptoethanol (Merck) and 50 µg/ml gentamicin (Sigma) at 37ºC for 24 hours. Supernatants were collected and stored at -20 ºC for cytokines analysis.

2.2.10. ELISA for total and GFP specific antibodies in serum and mucosal tissues

Polystyrene microtitre plates were coated overnight at 4ºC with 50 ng/well of purified goat anti mouse IgG (Immuno pure- Pierce biotechnology Inc) or rabbit anti mouse IgA (Immunology Consultant laboratories, New berg, OR, USA) or IgM (Bethyl laboratories, Montgomery, TX, USA) or IgE (Biolegend, San Diego, CA, USA) or recombinant GFP (Alpha diagnostic laboratories, San Antonio, USA) or PSA (Chemicon International) in PBS. Wells were blocked for 3 hours at room temperature using 3% BSA for IgG and 1hour at room temperature using 1% BSA for IgA, IgM, IgE and anti-GFP specific antibodies. Sera were tested at dilutions of 1/10,000 for IgG or 1/5000 for IgG_1 or 1/1000 for $IgG_{2a,}$ IgA, IgM or 1/100 for IgE or 1/500 for GFP antibodies. Fecal extracts or BAL were screened at dilutions of 1/500 for total IgA and 1/5 for GFP specific IgA. Intestinal fragment culture supernatant were also tested for total and GFP specific IgA.

Standard curves were produced using a dilution series of purified mouse IgG (Immuno pure- Pierce biotechnology Inc); IgG_1, IgG_{2a} (BD Pharmingen, San Diego, CA); IgA (Bethyl laboratories); IgM and IgE (BD Pharmingen, San Diego, CA.) and GFP or PSA monoclonal antibodies (Chemicon, USA). For GFP specific IgA ELISA ,GFP specific mouse IgA standard was not available, hence the values were expressed in Optical density (OD). Rabbit anti mouse IgG (DAKO, Glostrup, Genmark); IgG_1 and IgG_{2a} (Serotec, Kidlington, OX5 1GE, UK); goat anti mouse IgA (Immunology Consultant laboratories); IgM and IgE (Serotec) antibodies conjugated to (HRP) horseradish peroxidase was applied before color development using TMB substrate (Pierce biotechnology Inc). The absorbance was read at 450 nm; reference wavelength 570 nm (Tecan GENios/ Magellan).

At 80^{th} day of post primary nasal immunization, mice bladder, intestines and vaginal tissue were collected and homogenized in the presence of protease inhibitors (Protease inhibitor cocktail, Roche, Basel, Switzerland). Total protein and IgA concentrations were estimated using the Micro BCA protein assay kit (Pierce biotechnology Inc) and ELISA respectively.

2.2.11. Detection of anti *lactobacillus* antibodies

An extract of *lactobacillus* protein was prepared as previously described for *Lactococci* (Wells *et al.* 1993). *Lactobacilli* were recovered from MRS medium by centrifugation following addition of NaCl to a final concentration of 1M. Cells were washed three times with wash buffer (100 mM Tris-HCl pH7.5, 5 mM $MgCl_2$, 2mM EDTA, 1 mM PMSF) and each gram of cells (wet weight)

suspended in about 3-4 ml of ice cold wash buffer containing protease inhibitors (2 μM leupeptin,1 μM pepstatin A and 0.1 mM PMSF). The cells were homogenized with 40-50 g of glass beads (0.10 mm diameter) for 2 x 30s. The glass beads were removed by glass filter and the homogenate centrifuged at 10,000 x g for 15 min to pellet the cell walls. The membranes were removed from soluble protein fraction by centrifugation at 144000 x g for 75 min at 4º C. ELISA plates were coated overnight with 100 ng/well of membrane extract in PBS at 4ºC and then blocked for 1 hour at room temperature with 1% BSA (Sigma). Serum, BAL fluid and fecal extract were tested for LGG specific antibodies. To compare the specific and non specific antibody induction the high antibody titer serum collected on day 35 was serially diluted to obtain approximately the same absorbance for both anti-LGG and anti-GFP ELISA and the titer differences were calculated by comparing the serum dilution factors.

2.2.12. Cytokine analysis of BAL and intestinal fragment culture supernatant

Cytokines (TNFα, IFNγ, IL2, IL4, IL6, IL10, IL12 and TGFβ) were analyzed with commercial ELISA kits (BD Biosciences or eBiosciences). Briefly, 100 μl capture antibody (Ab) diluted in coating buffer was added to each well, and incubated overnight (4^{o}C). The plates were then washed three times with wash buffer (PBS with 0.05% v/v Tween 20) and blocked with 200 μl assay diluent (RT, 1 hour). Following which, the plates were washed 3 times before the addition of 100 μl standard or sample. Plates were incubated for 2 hour at RT. The plates were washed again, then 100 μl working detector (biotinylated detection Ab and

strepavidin-horseradish peroxidase conjugate (HRP) was added to each well (RT, 1hour). The plates were then washed before 100 µl TMB substrate solution (Pierce Biotechnology Inc., USA) was added to each well, and incubated in the dark (RT, 30 min). 50 µl stop solution (2N H_2SO_4) was added to each well to stop the reaction. The absorbance was read at 450 nm; reference wavelength 570 nm (Tecan GENios/ Magellan).

2.2.13. Visualization of the bacteria after oral or nasal immunization

2.2.13.1. Confocal or electron microscopy

Twenty four hours after oral immunization, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg) and perfused with a warm PBS followed by freshly prepared fixative mixture (2% paraformaldehyde + 3% glutaraldehyde) in a buffer system for 15 minutes. After that, mice small intestines, cecum and large intestines were removed and some of them were snap frozen in isopentane (Sigma Aldrich) that was prechilled in liquid nitrogen. Cryosections (5-10 µm) of the specimens were directly visualized for green fluorescence under confocal microscopy (Carl Zeiss). For Transmission electron microscopy (TEM), tissues were cut into small pieces of 1 mm^3 and immersed in the fixative (2% paraformaldehyde + 3% glutaraldehyde) for 2 - 4 hours at 4º C. Thereafter, tissues were rinsed in the same buffer (5% sucrose) and post fixed in 1 to 2% OsO_4 ,pH 7.4 for 1 hour at room temperature. After washing in the buffer for 5 - 10 minutes, samples were dehydrated (25% ethanol, 5 min; 50% ethanol, 10 min; 75% ethanol, 10 min; 95% ethanol, 10 min; 100% ethanol

10 min). After dehydration, the samples were transferred to 100% Acetone 10 min (twice with fresh acetone each time). The samples were incubated in ethanol-araldite (1: 1) solution for 15 min, then overnight at room temperature in ethanol-araldite (1: 6) solution. The following day, the samples were incubated for 1 hour each in 100% araldite at 40^{o}C, 45^{o}C, and 50^{o}C. Finally the samples were embedded in pure araldite (60^{o}C for 24 hours), then trimmed and sectioned with an Ultramicrotome (~80 nm thick sections) and viewed with an electron microscope (Phillips, Netherlands).

2.2.13.2. Bacterial uptake *in situ*

4-6 weeks old, female C57BL/6 mice were anesthetized by intra peritoneal injection of 100 mg/kg of ketamine and xylazine (9 mg/kg) per mouse. Around 10 cm long segments of the small intestine of mice were ligated at both ends with surgical thread as described by Jang *et al.* 2004. GFP-expressing LGG (1 x 10^{8} CFU) were suspended in 1.0 ml of sterile PBS and inoculated into the loop and incubated *in situ*. One hour later, intestinal segments (without PP) were removed and extensively washed with cold PBS and RPMI medium 1640 containing gentamycin (100 μg/ml). Whole mounted intestinal segments were fixed in 4% paraformaldehyde followed by washing in 10% FBS in PBS, and M cells and columnar epithelial cells were stained with rhodamine conjugated Ulex europaeus agglutinin (UEA-1, Vector laboratories) and Alexa fluor 633 conjugated Wheat germ agglutinin (WGA, Molecular probes, Invitrogen) respectively. Some intestinal parts were cryosectioned and stained for M cells or columnal epithelial

cells as described above. Tissue sections or whole-mounted small intestinal segments were viewed under confocal microscopy (Carl Zeiss).

2.2.13.3. Tracking of GFP expressing LGG in lung after 24 hrs of nasal immunization

Mice were euthanized one day after nasal immunization with LGG-GFP and lungs were harvested and cut into small pieces before being snap frozen in isopentane that was pre-chilled in liquid nitrogen. About 10 μm size cryosections were made and air dried on poly L-lysine coated slides. The slides were blocked in 3% H_2O_2 dissolved in methanol for 15 min to inhibit the endoperoxidase activity. After blocking, the slides were washed then blocked in 5% normal rabbit serum for 60 mins; washed and incubated with anti-GFP monoclonal antibody (Abcam Inc, Cambridge, USA) overnight at 4ºC (1:500). The next day, the slides were washed before incubation with horseradish peroxidase labeled rabbit anti-mouse IgG (1:1000 dilution) for 1 hr. The slides were washed three times in 1 x PBS (5 min per wash) before a final rinse with Tris-Buffered Saline (TBS). Following which the slides were incubated in DAB solution for 10 mins then washed 5 times in TBS (5 min per wash). The slides were counterstained with methyl green for 5 min and then rinsed in ddH_2O. Finally the stained slides were dehydrated in alcohol, cleared in Histoclear and mounted with a coverslip.

2.3 Ex vivo experiments

The murine transitional cell carcinoma cell line, MB49 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone), 2 mmol/L L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen) at 37^{o}C and 5% CO_2. MB49-PSA or MB49-GFP cells, were MB49 cells stably transfected with the human prostate specific antigen (PSA) or GFP (Wu *et al.* 2004) and were maintained as described above with 0.2 mg/ml hygromycin (Invitrogen).

2.3.1. Generation and purification of bone marrow-derived dendritic cells (BMDC)

The procedure used to generate DCs from bone marrow cultures was as described previously by Mayordomo *et al.* 1995 with minor modifications. Briefly, the bone marrow was flushed from the long bones of the limbs of C57BL/6 mice, filtered through a 70 μm cell strainer, and depleted of red cells with RBC lysis buffer. Bone marrow cells were plated in a culture dish (1.5 x 10^6 cells/ml; 10 ml/dish) in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol (ME), 1% penicillin, streptomycin, glutamine, MEM (Minimum essential medium), and 0.1% sodium pyruvate. Recombinant Murine Granulocyte Macrophage Colony Stimulating Factor (GM-CSF, eBiosciences) was added at a concentration of 20 ng/ml. Every 3-4 days, the culture media was changed and fresh mGM-CSF was added. On days 8–10 DC were harvested by gentle pipetting. For purification, cells were centrifuged at 200 x g for 10 minutes and resuspended in 400 μl MACS buffer (PBS, pH 7.2, supplemented with 0.5% BSA

and 2 mM EDTA). To obtain high purity, Fc receptor blocking was done (CD16/32 antibody – Miltenyi biotec, Bergisch Gladbach, Germany) before adding 100 µl of CD11c microbeads (Miltenyi biotec). After CD11c microbeads were added, cells were mixed well and incubated for 15 minutes at 4 - 8°C. Cells were washed by adding 1-2ml of MACS buffer per 10^8 cells and centrifuged at 200 x g for 10 minutes. The supernatant was removed and cells were resuspended in 500 µl of buffer. A MS column was placed in the magnetic field of a suitable MACS Separator and rinsed with 500 µl of MACS buffer. The cell suspension was applied in 500 µl to 1ml volume and after it passed through, the column was washed with 3 x 500 µl of MACS buffer. Retained cells were eluted outside of the magnetic field and collected as dendritic cells. The purity of the dendritic cells were analyzed by flow cytometry BD FACS Canto (Beckton Dickinson, San Jose, CA) and FACSDiva software (Fig.2.2.b).

2.3.2. Murine bone marrow neutrophils (BMN) purification

Neutrophil purification from bone marrow cultures was done as described before with some modifications (Beauvillain *et al.* 2007). The bone marrow was flushed from the long bones of the C57BL/6 mice limbs, filtered through a 70 µm cell strainer, and depleted of red cells with RBC lysis buffer. Cells were spun down at 2000 rpm for 5 mins and the supernatant was discarded. The cell pellet was resuspended in 200 µl of MACS buffer (cell count $<10^8$ for MS column). and 50 µl of Anti-Ly6G Biotin was added (Miltenyi biotech) and mixed well, followed by incubation at 4 - 8^oC for 10 mins. Then 150 µl of MACS buffer and

100 µl of Anti-Biotin Microbeads were added. Cells were mixed well and incubated for 15 minutes at 4 - 8^{o}C. Cells were washed by adding 5-10ml of MACS buffer followed by centrifugation at 300 x g for 10 mins. The supernatant was removed completely and the pellet resuspended in 500 µl of buffer and loaded on a rinsed MS column was placed in the magnetic field of a suitable MACS Separator. The column was washed with MACS buffer (3 x 500µl). Retained cells (neutrophils) were eluted outside of the magnetic field with 500 µl of MACS buffer. Cells were stained with FITC labeled Ly6G antibody and analyzed by flow cytometry (Fig 2.2.a)

2.3.3. Bacteria – DC or neutrophils co-culture

BMDC or bone marrow derived neutrophils (5 x 10^5) were plated in 24 well plates in RPMI medium supplemented with 2Mm L-glutamine, 50U/ml of penicillin, 50 µM β-mercaptoethanol (sigma), 10% FCS and 10ng/ml of GM-CSF (eBiosciences). Cells were incubated with recombinant LGG carrying IL2 or IL15 or IL7 with or without PSA for 24 hours at a bacteria to cell ratio of 100:1. Cells were washed in PBS three times and stained with the mAbs directed against the surface molecules CD86, CD80, CD83, CD11c, MHC II, and CD40 or appropriate isotype-matched controls. Neutrophils were stained for CD86, CD83, CD11b or appropriate isotype controls. Analysis was performed with BD FACS Diva^{TM} software. Supernatants from bacteria-DC or neutrophils co-culture were harvested and analyzed by ELISA for mouse IL2, IL6, IL12p70, IL10, TNFα, and IFNγ .

2.3.4. Induction of PSA specific primary T cells in vitro

PSA specific primary T cells were generated as described before with some modifications (Rouse *et al.* 1994). T lymphocytes from naive C57BL/6 mice spleen were enriched by using STEM CELL-Easy sep^{R} T cell isolation kit (Stem cell technologies, Vancouver, BC, Canada). A single cell suspension was prepared from spleen after mechanical disruption at a concentration of $1x10^{8}$ cells/ml in medium (PBS with 2% FBS and 5% normal rat serum). Cells were placed in a 5 ml (12 x 75 mm) polystyrene tube or polystyrene round bottom tube (BD biosciences) to properly fit into the purple EasySep magnet. EasySep mouse T cell enrichment cocktail at 50 µl/ml of cells (e.g. for 2 ml of cells 100 µl of cocktail) was added to the cells and incubated at 4° C for 15 minutes. Then biotin selection cocktail at 100 µl /ml cells was added followed by gentle mixing. Sample was incubated at 4°C for 15 mins. Magnetic nanoparticles were mixed to ensure that they are in uniform suspension by pipetting vigorously for 5 mins. Nanoparticles were added at 50 µl /ml cells followed by incubation at 4°C for 15 mins. The cell suspension total volume was brought to 2.5 ml by adding the recommended medium without rat serum. Cells were mixed by gentle pipetting up and down 2 - 3 times. Then the tube was placed into the magnet (without cap) and left for 5 minutes. The magnet and tube were lifted and inverted (2 - 3s) to collect the fraction of cells that were not bound, in a new 5 ml polystyrene tube. The magnetically labeled unwanted cells remained bound inside the original tube, held by the magnetic field of the magnet. Usually the desired fraction was about 95 - 98% CD3 positive as seen in Fig. 2.2.c. Purified

T cells (1.0 x 10^7 cells per ml) were cultured with DC infected (1.0 x $l0^5$ cells per ml) with PSA secreting recombinant LGG to give responder-to-stimulator ratio-100:1 in 200 µl of LDA medium (NCTC 109 and RPMI 1640 [1:1]), supplemented with 10% heat-inactivated FCS, 10 mM Lglutamine, 1 mM oxalacetic acid, 0.2 U of bovine insulin per ml, and 50 µM of 2-mercaptoethanol in 96-well U-bottom plates. The plates were incubated at 37°C under 5% CO_2 . For positive control, splenocytes from naive mice (60 x 10^6 cells per flask) were incubated with 5 µg/ml of PSA specific CTL peptide (VISNDVCAQV, Proimmune, UK). After 5 days, cells were used as effector cells for antigen presentation assay or CTL (cytotoxic lymphocyte) assay.

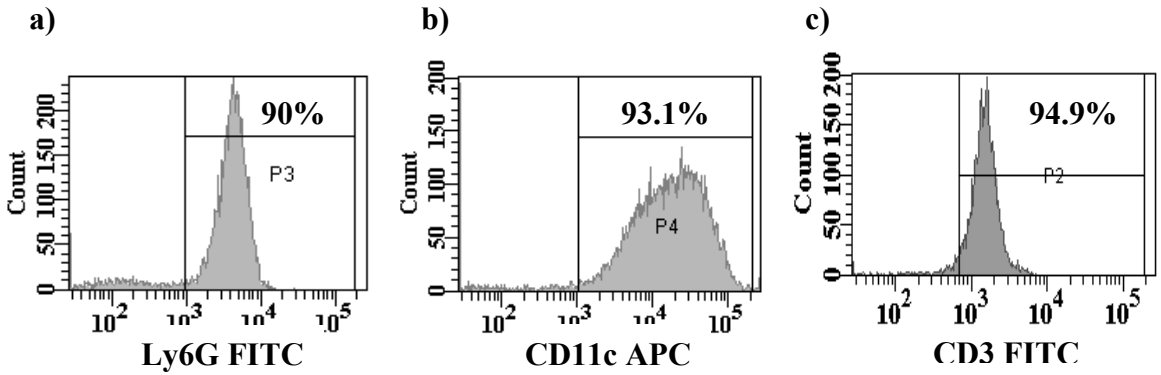


Figure 2.2. Purity of the cells used for the bacteria stimulation experiments

a) Purity of the bone marrow derived neutrophils after positively enriched by using Ly6G microbeads. b) Purity of bone marrow derived dendritic cells harvested on 9th day of culture. c) Purity of the CD3^{+} T cells enriched from the mice spleen by negative selection.

2.3.5. CTL and antigen presentation assays

The antigen presentation assay was done as described before with some modifications (Cheadle *et al.* 2005). Dendritic cells (5 x 10^5) were plated in 24 well plate (total volume 2 ml and infected with recombinant LGG secreting IL2 or IL15 or IL7 or PSA or IL2-PSA or IL15-PSA and or IL7-PSA at a bacteria to cell ratio of 100:1 for 24 hours. After gentamycin treatment (200 μg/ml for 2 hours) and three washes in PBS, 2 x 10^4 DCs were cultured with 5 x 10^5 purified T cells from naïve mice in 96 well U-bottom plates to give responder to stimulator ratio of 25:1. After 5 days, 100 μl of medium was removed for IL2 and IFNγ detection. For CTL assay 100 μl of medium was removed and 100 μl of target cells were added and incubated for 5 hours. Syngenic mice splenocytes pulsed with PSA CTL peptide (2 - 5 hours) or MB49-PSA were used as target cells. The supernatant were harvested and LDH activity was determined by using CytoTox 96^{TM} kit (Promega, Madison, WI, USA). The mean percentage of specific lysis of quadruplicate wells was determined using the following formula: % of cytotoxicity = [(experimental – effector spontaneous-target spontaneous) / Target maximum-target spontaneous)] x 100.

2.3.6. *Ex vivo* ELISPOT assay

Ex vivo ELISPOT assay was done as described before with some modification (Parent *et al.* 2005). On the 80th day, intranasally or orally immunized mice were euthanized and splenocytes were harvested. $CD4^+$ T cells were positively enriched using CD4 microbeads (Miltenyi Biotec). The resultant cells were >85% $CD4^+$ as determined by flow cytometry. 1 x 10^5 $CD4^+$ T cells in DMEM

complete medium (Dulbecco's Modified Eagle medium) were plated on mouse IFNγ ELISPOT plates (BD Pharmingen). Each well also contained 1 x 10^5 mitomycin C (Sigma) pretreated splenocytes (50 μg/ml) harvested from PBS administered mice and 10U of IL-2/ml. The plates were incubated at 37ºC with 5% CO_2 for 48 hours with or without 1μg of recombinant GFP. For CD8 ELISPOT, plates were loaded with 1 x 10^6 splenocytes per well, with GFP specific CTL peptide (H-2Kd HYLSTQSAL) (1 μg/ml) and 1 x 10^5 mitomycin C treated control splenocytes. Then the plates were incubated for 48 hours. The antigen specific IFN-γ secreting CD4 or CD8 T cells were visualized and enumerated.

2.3.7. CTL response against MB49-GFP tumour cells

CTL activity of spelnocytes from immunized mice was determined by measuring lactate dehydrogenase (LDH) activity released from lysed cells (Cytotox 96R, Promega). Briefly, 1 x 10^6 spleen cells were co-cultured with 1 x 10^6 mitomycin-C treated MB49-GFP cells and 1 μg/ml of GFP specific CTL peptide (H-2Kd HYLSTQSAL, Proimmune, UK) in RPMI 1640 supplemented with 5% FBS, 50U/ ml penicillin, and 50 u/ml of streptomycin for 5 days. The splenocytes were recovered and used as effector cells against 1 x 10^4 viable MB49-GFP cells as target cells at a ratio of 1:1, 10:1 and 30:1. After 5 hours of culture, supernatants were recovered and LDH activity was determined. The mean percentage of specific lysis of quadruplicate wells was determined using the following formula: % of cytotoxicity = [(experimental – effector spontaneous-target spontaneous) / Target maximum-target spontaneous)] x 100

2.4. Statistical analysis

All animal experiments were independently replicated at least twice whereas *in vitro* experiments were independently replicated at least thrice, each time in duplicates. Graphs were prepared using GraphPad Prism and statistical analysis was performed using one way ANOVA with post hoc Scheffe test (SPSS software). A p value of < 0.05 was taken to indicate a significant difference. All graphical data was expressed as the mean ± SD (unless stated otherwise) of combined data from the replicate experiments.

Chapter Three Results

3.1. Expression of the model antigen GFP with a cytokine in LGG

The specific aim of this dissertation was to explore the potential of *Lactobacillus rhamnosus* GG (LGG) as antigen delivery vehicle. A model antigen GFP was expressed in LGG and to enhance the immune response, murine IL2 was also co-expressed with the antigen. GFP was cloned in the *E .col i- Lactobacillus* shuttle vector, pLP500 under the control of the *ldh* promoter with or without murine IL2. To study the general or specific immune response elicited by mucosal immunization, mice were immunized orally or intranasally with LGG secreting GFP or IL2-GFP. Wild type LGG or PBS was administered as controls.

3.1.2. Expression or co-expression of model antigen, GFP with murine IL2

The supernatant from overnight cultures of LGG-GFP or LGG IL-2-GFP were collected to assess GFP and IL2 secretion and the bacteria were collected for analysis of bacterial numbers by plating. GFP expression in LGG-GFP and LGG-IL2-GFP was confirmed by flow cytometry and confocal microscopy (Figure 3.1).

As the GFP expression in LGG-GFP or LGG-IL2-GFP was almost similar, either flow cytometry or confocal microscopy picture was only shown for GFP expression.

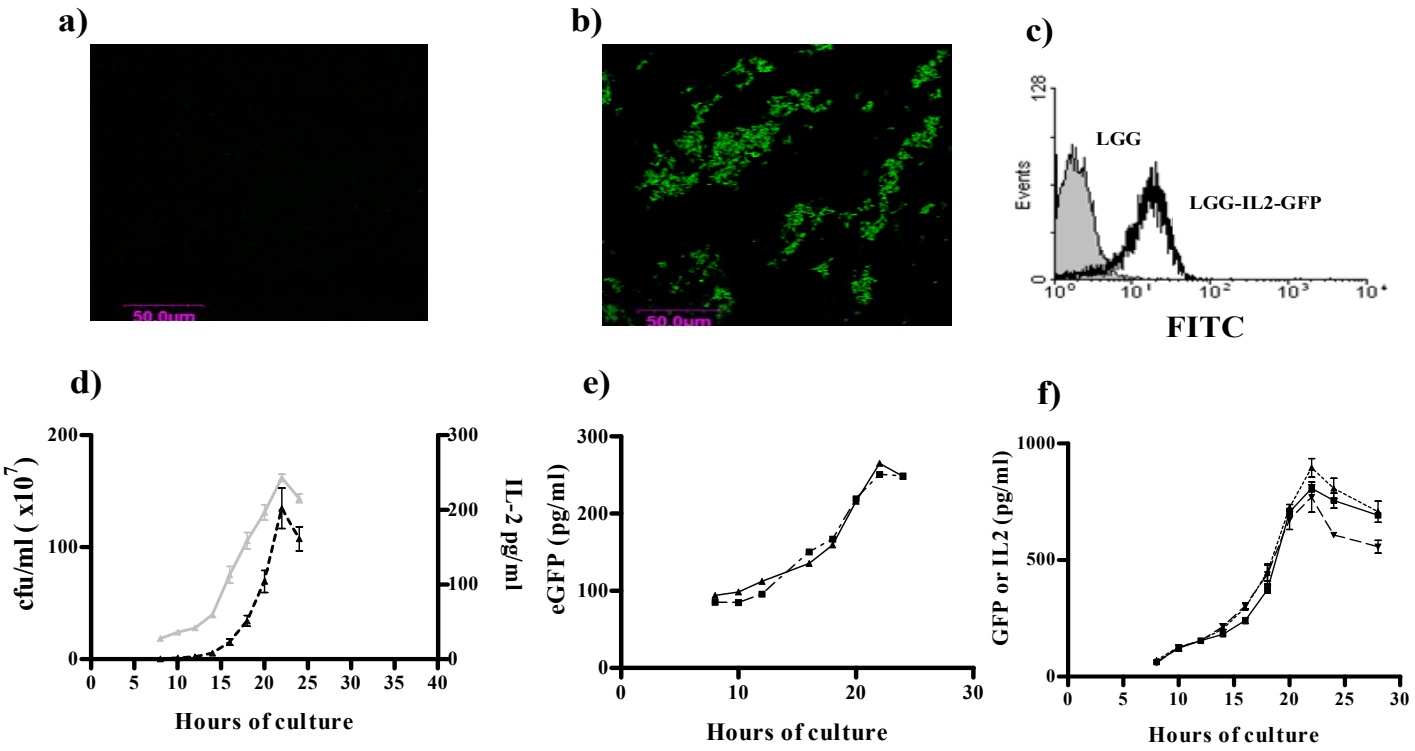


Figure 3.1. GFP or IL2 expression in modified LGG (LGG-GFP or LGG-IL2-GFP)

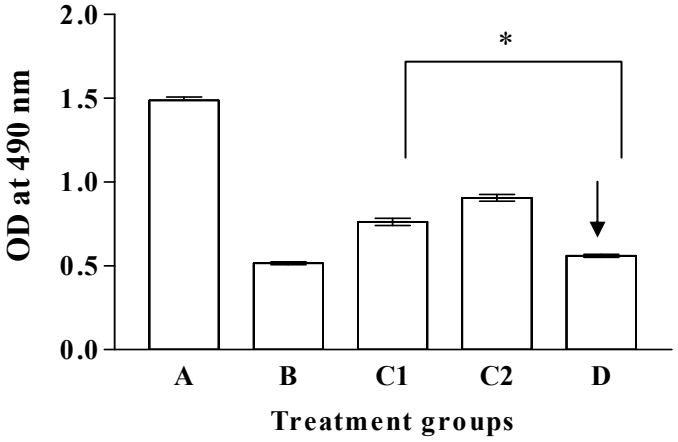
GFP fluorescence was visualized under confocal microscopy (scale bar 50 µm), a) control wild type LGG and b) LGG-GFP; c) Analysis of GFP expression in LGG-IL2-GFP by flow cyometry; d) Secretion of IL-2 vs bacterial cfu with time, the dashed line represents cfu/ml of bacteria and the solid line represents IL-2 secreted into the supernatant in pg/ml; e) Secretion of GFP in the culture supernatant- the dashed line represents LGG-GFP and solid line represents LGG-IL2-GFP and f) Intracellular expression of GFP or IL2- the dotted or solid line represents GFP or IL2 expression in LGG-IL2-GFP respectively and the dashed line represents GFP expression in LGG-GFP.

Even though, the plasmid has a secretory signal sequence from the *prt* P gene of *L. casei* (Ho *et al.* 2005) for secretable expression, there was a significantly detectable level of internal expression as well. The levels of IL-2 and GFP secreted in the culture media increased with bacterial CFU reaching a maximum at about 22 hours when the bacteria were at the end of the exponential phase and decreasing thereafter (Figure 3.1.d). Therefore the bacteria used for immunization were harvested after 22 hours of culture. At this point the total production (intracellular and secretory) of IL-2 and GFP was about 1.143 and 1.072 ng/ml respectively from 1.3 x 10^9 CFU/ml of LGG-IL2-GFP. GFP concentration in LGG-GFP was about 1.021 ng/ml at 22 hours of culture (Fig 3.1.f). Only 1/5 of the total protein produced was secreted and the secretion of GFP from LGG-IL2-GFP and LGG-GFP was 248 and 251 pg/ml respectively and the amount of IL2 secreted by LGG-IL2-GFP was 265 pg/ml as seen in fig. 3.1.e.

3.1.3. IL2 secreted by LGG-IL2-GFP is biologically active

To analyze the biological activity of IL2 secreted by LGG-IL2-GFP, a CTLL-2 proliferation assay was performed. Culture supernatants from LGG-IL2-GFP was harvested after 22 hours of culture and dialyzed against blank RPMI followed by ultra filtration to concentrate the protein. FBS was added to get 10% FBS in RPMI and sterile filtered. CTLL-2 cells were harvested from stock cultures supplemented with IL-2 and washed twice by centrifugation. CTLL-2 cells were dispensed into 96 well assay plates (5 x 10^3 cells/well) and cultured for 20-24 hours in the presence of IL-2 or bacterial culture supernatant. Various concentrations of Human Recombinant IL-2 were used as

standard. To confirm the IL2 mediated proliferation, a blocking antibody to IL2Rα was added with bacterial culture supernatant. After 1 day of culture, 20µl of CellTiter 96® AQueous One Solution Reagent, was added and the assay plates were incubated for an additional 4 hour period. 490nm absorbance was recorded directly from the CellTiter 96® Assay plate. The bioactivity of IL-2 fused to GFP was determined to be 3.244 U/ml of culture supernatant (Figure 3.2).



Treatment groups	recombinant IL2 (1µg/ml)	LGG-IL2-GFP culture supernatant		LGG-IL2-GFP culture supernatant (C1) + IL2Rα antibody
		C1 (100ng/ml)	C2 (200ng/ml)	
A	+	-	-	-
B	-	-	-	-
C (C1/C2)	-	+	+	-
D	-	-	-	+

Figure 3.2. CTLL-2 proliferation assay. IL2-GFP-LGGculture supernatant was dialyzed against blank RPMI and concentrated by ultra filtration before determining its biological activity with a CTLL-2 proliferation assay. Media without IL2 was used as blank. IL2 induced proliferation was abolished in the presence of the blocking antibody to IL2Rα (arrow). * Statistically significant (p value <0.05).

3.1.4. Stability of transformed bacteria

The heterologous protein expression depends on the stability of the recombinant plasmid. Culturing recombinant bacteria in antibiotic selection ensures the survival of bacteria which possess the recombinant plasmid. Recombinant LGG may loose the plasmid at mucosal sites, since antibiotic selection is not possible *in vivo*.

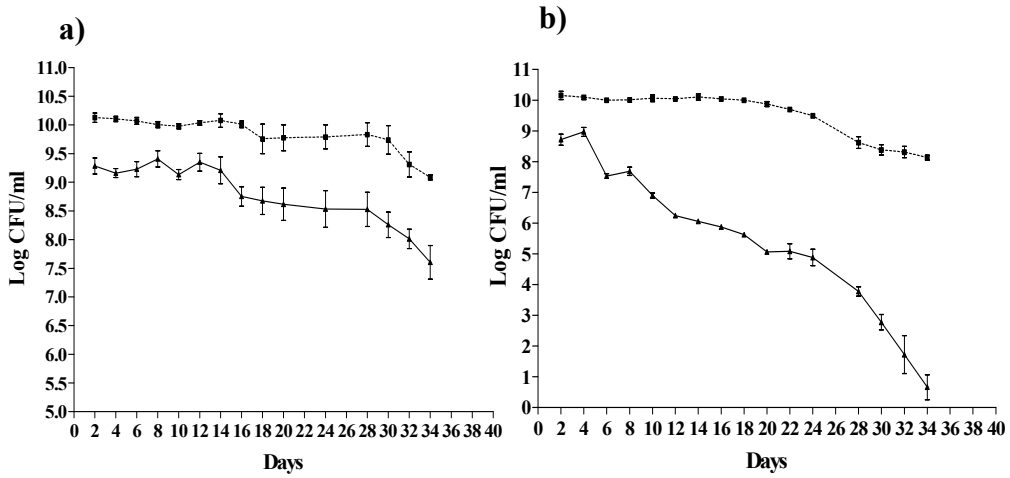


Figure. 3.3. Divergent stability of recombinant plasmids in LGG in non selective environment. LGG secreting GFP (a) or IL2-GFP (b) were subcultured every day in MRS broth with (dotted line) or without (solid line) erythromycin. Bacterial CFU in the daily culture was determined by plating bacteria on MRS agar plates with eryhtromycin.

Though booster immunization may be helpful in colonization of recombinant LGG at mucosal sites, plasmid stability in a non-selective environment would determine the continuous antigen production from the bacteria at colonizing sites. We analyzed the stability of IL2-GFP or GFP secreting LGG in a non-selective environment by sub- culturing the bacteria every day in MRS broth with or without erythromycin and plating the bacteria on ery^{+} MRS plates to determine the number of bacteria that still have the plasmids. Though

continuous propagation in non-selective media caused gradual plasmid loss both in GFP or IL2-GFP secreting LGG, the pLP500-GFP plasmid was more stable than the pLP500-IL2-GFP plasmid (Figure 3. 3). However, there was a 50% reduction in bacteria carrying the IL-2-GFP plasmid only after 20 days of continuos culture.

3.2. Survival and colonization ability of LGG after oral or nasal immunization.

The main advantage of using a *Lactobacillus* based vaccine is the ability of lactobacilli to colonize the mucosa. Therefore bacterial localization in lung or intestines; bacterial translocation to draining lymph nodes or spleen or liver and persistence after nasal and oral immunization were examined.

3.2.1. Translocation of modified LGG after nasal or oral immunization

To locate Lactobacilli after oral or nasal immunization with $1x10^8$ CFU of bacteria the MLN (oral immunization) or NALT, CLN and Med.LN (nasal immunization) were collected. For the analysis of systemic translocation, the spleen and liver were also collected. The tissues were homogenized and bacterial cfu determined by quantitative plating on Ery^+ MRS agar plates. Bacterial load was determined at 24 hours, 48 hours and 1 week post-immunization. Twenty-four hours after intranasal immunization, more bacteria were found in the NALT than CLN or Med.LN (Table 3.1). These bacteria were progressively and completely cleared from the NALT, CLN and Med.LN and no more bacteria were found after 1 week. No bacteria were detected in

the spleen or liver. Both modified Lactobacilli showed the same pattern of translocation.

After oral immunization many bacteria were found to be translocated to MLN. IL2-GFP secreting LGG translocated in higher numbers to the MLN after 24 and were still present in significantly higher number after 48 hours (Table 3.1).

Table 3.1 Translocation of recombinant LGG after oral or intranasal immunization

	LGG expressing	Bacterial cfu/gm of tissue								
Oral administration		MLN			Spleen			Liver		
		24 hrs	48 hrs	168 hrs (1 week)	24 hrs	48 hrs	168 hrs (1 week)	24 hrs	48 hrs	168 hrs (1 week)
	GFP	96 ± 48	0	0	7 ± 11	0	0	15 ± 3	0	0
	GFP- IL2	1308 ± 501*	67 ± 9*	0	78 ± 41	15 ± 4	0	45±13	0	0
Intranasal administration		NALT			CLN			Med.LN		
		24 hrs	48 hrs	168 hrs (1 week)	24 hrs	48 hrs	168 hrs (1 week)	24 hrs	48 hrs	168 hrs (1 week)
	GFP	9944 ± 1523	144 ± 132	0	37 ± 11	0	0	110 ± 40	0	0
	GFP-IL2	10052 ± 1259	140 ± 96	0	37 ± 12	0	0	114 ± 31	0	0

Data represent mean cfu ± standard deviations

* Statistically significant ($p < 0.05$)

3.2.2. Persistence of modified LGG after oral immunization at gut on 80th day.

Lactobacilli were known to colonize the intestine. We examined LGG colonization at the end of the immunization schedule. Different colonization ability of LGG-GFP or LGG-IL2-GFP in the small intestines were analyzed on the 80th day post primary immunization (52 days after the last immunization) by quantitative plating (Table. 3.4).

Table 3.2. Different colonizing ability of recombinant LGG in oral immunization.

Immunization	Bacteria CFU/ml of small intestine homogenate		
	duodenum	jejunum	ileum
LGG (pLP500) LGG-GFP LGG-IL2-GFP	131 ± 32* 144 ± 42* 21 ± 8	38 ± 19 56 ± 24 0	12980 ± 1230* 12120 ± 850* 967 ± 117

Data represent mean ± standard deviations
* Statistically significant ($p < 0.05$) compared to LGG-IL2-GFP.

3.3. Tracking of recombinant LGG using GFP as visible marker in immunization

Direct observation of the GFP expressing bacteria by fluorescence microscopy (Figure 3.4, a - d); immunohistochemical staining (Figure 3.4, e-h) and Transmission Electron Microscopy (Figure 3.5, a-f) allow us to trace bacteria in intestinal sections or lung sections of mice fed with GFP or IL2-GFP expressing LGG. Though quantitative analysis was not done, It also will be possible to analyze the interaction of LGG with M cells by using a ligated-intestinal-loop system to avoid *in vivo* dilution of the sample.

Immunohistochemical staining of LGG in lung and confocal microscopic examination of intestine showed the bacterial persistence in lung or intestine. Transmission electron microcopic examination clearly showed the bacterial uptake in intestine. (Figure 3.5, a-c). However many bacteria especially IL2-GFP secreting LGG were seen in intravascular region which was an uncharacteristic for *lactobacilli* (Figure 3.5, e&f). Orally immunized bacteria are known to be taken up by M cells at peyers patches (Macpherson *et al.* 2004). However in our TEM study the bacterial uptake was found to be mediated through intestinal epithelial cells. Hence LGG uptake by M cells at intestinal villus was investigated by loop ligation method as described previously for *Salmonella typhimurium* (Jang *et al.* 2004).

Under anaesthesia mouse intestine was ligated near caecum and 10cm from caecum and bacteria was injected (LGG-GFP - 10^8 cfu) in to intestine. After 1 hour mice were euthanized and intestine was harvested and stained for M cells and FAE. As seen in Figure. 3.6, few GFP fluorescing LGG were co-localized with M cells at intestinal villi which suggested M cell mediated LGG uptake at intestinal epithelium (Figure. 3.6)

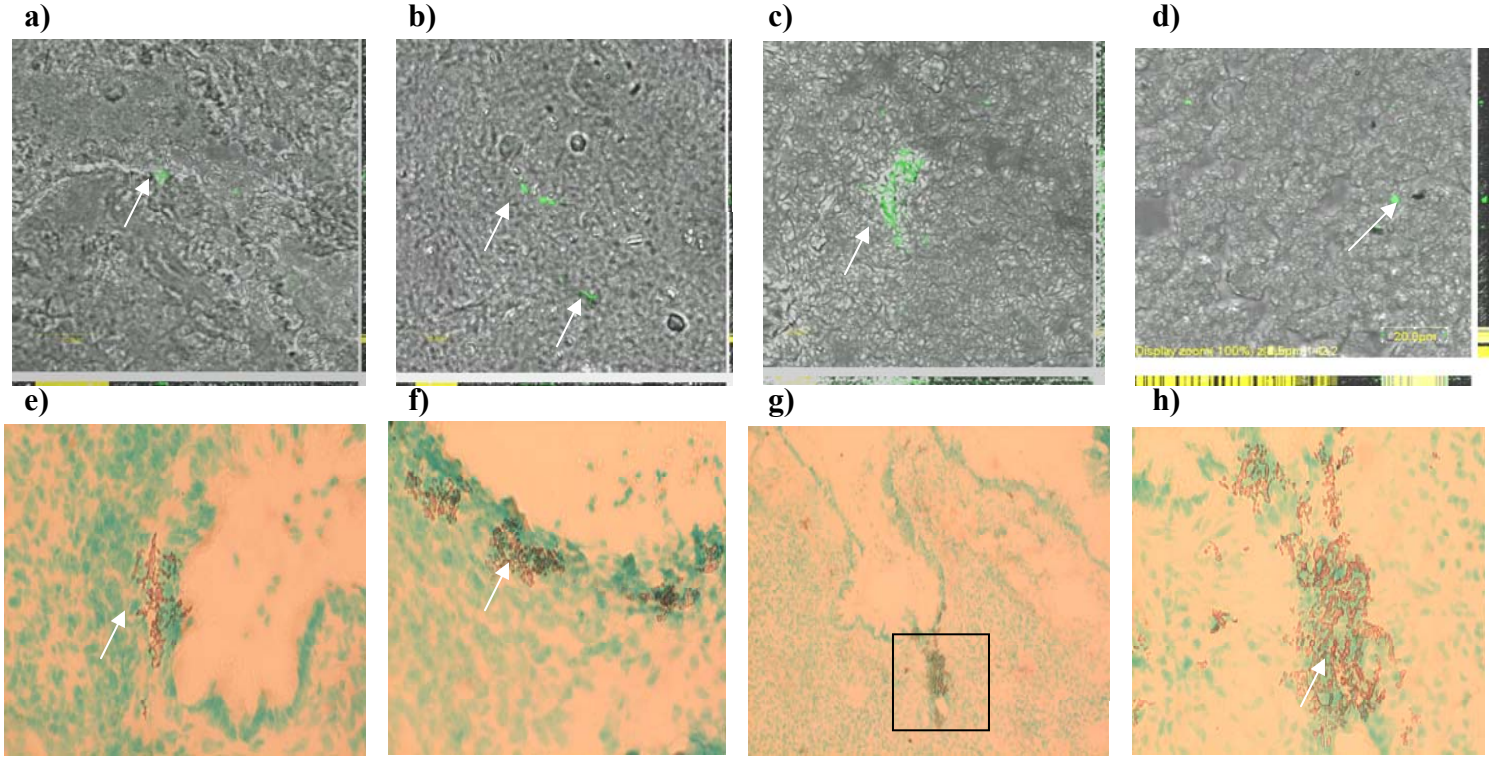


Figure 3.4. Tracking of LGG-GFP (a,b & e,f) or LGG-IL2-GFP (c,d & g,h) in mice intestine (a-d) and lung (e-h) 24hours after of oral or intranasal immunization. Cryosections of orally immunized mice caecum (a-c) or small intestines (d) were examined under confocal microscopy and compressed Z-stack of optical sections were taken. Paraffin sections of nasally immunized mice lungs were stained with Horseradish Peroxidase labeled anti-GFP antibody followed by detection with diaminobenzidine (DAB) substrate. Bacteria were stained brown and were observed in the bronchiolar linings. Figure (h) is the magnified view of the boxed region in figure (g). The scale bar for a) is 20 µm; for b and c it is 10 µm and for d it is 20 µm. For e, f, h the magnification is 2 x 20 and for g, the magnification is 2 x 10.

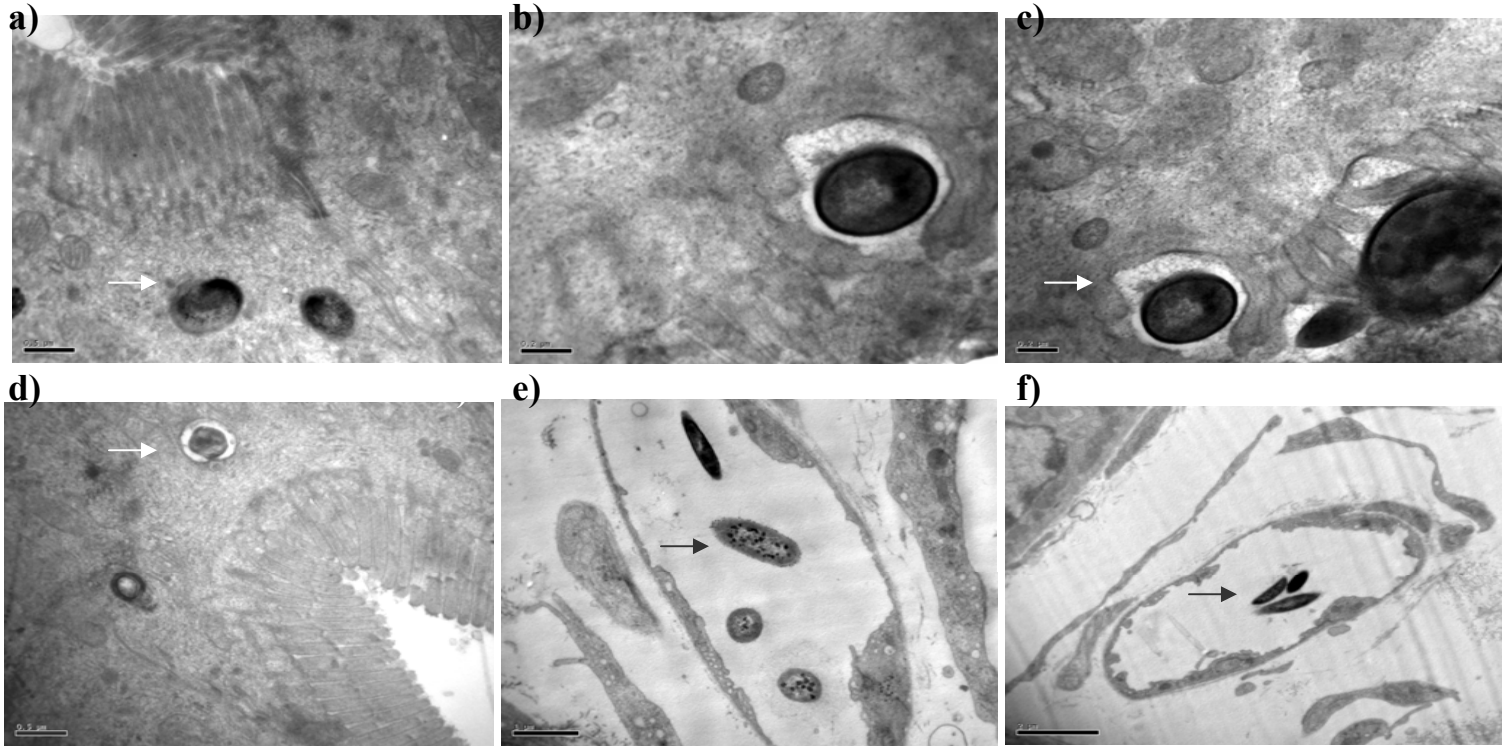


Figure 3.5. Visualization of recombinant LGG in mice intestine ultrathin sections 24 hrs after oral administration by Transmission Electron Microscopy (TEM). Mice intestines were sterilized with ampicillin by oral administration followed by immunization with 10^8 CFU recombinant LGG. After 24hrs, the small intestine, large intestine and caecum were processed and examined for LGG-GFP (a-c) or LGG-IL2-GFP (d-f) under TEM. The scale bar for a and b is 0.2 μm; for c and d it is 0.5 μm and for e and f it is 1 μm. Arrows indicate the uptake (a, c, d) and the presence of bacteria in the intravascular region (e and f).

3.3.1. Bacterial uptake in mice intestinal villus

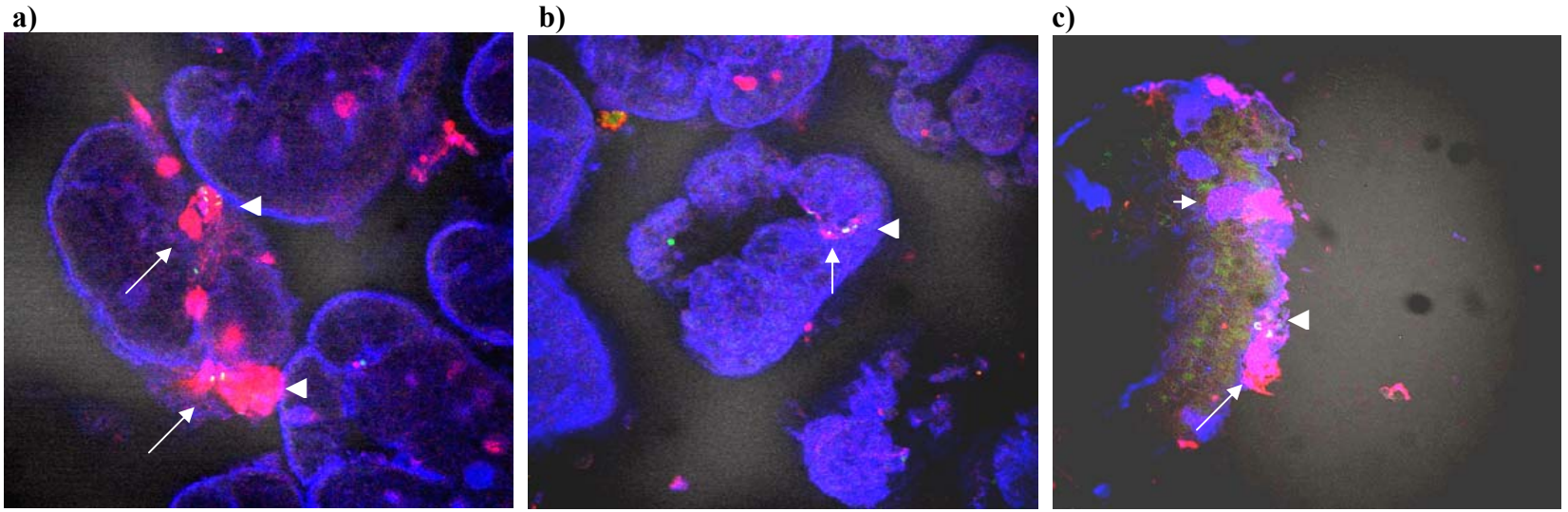


Figure 3.6. Confocal microscopic view of villous epithelium. Villus epithelium was stained for M cell and columnar epithelial cell-with UEA-1-TRITC (Ulex europaeus agglutinin) and WGA- Alexa fluor 633 (Wheat germ agglutinin) respectively. Whole mount preparation of small intestine (a & b) or cryosection of the intestinal segment (c). M Cells were stained by UEA-1 (red, arrow), enterocytes by WGA (blue), and goblet cells by UEA-1 and WGA (red-blue mix, arrowhead). LGG-GFP (a&b) or LGG-IL2-GFP (c) were seen co-localized with M cells(◄). The scale bar for a and b is 20 μm; for c 10 μm.

Summary I

Though the recombinant LGG were able to secrete GFP or IL2-GFP, the level of secretion was about 240 pg only. Moreover the secretion was not 100% and the majority of the recombinant protein was intracellular. However IL2 secreted as IL2-GFP fusion protein by recombinant LGG was biologically active. Recombinant LGG were able to colonize the intestine and lung after oral or nasal immunization. So the colonized recombinant LGG would secrete antigen which will be continuously taken up by dendritic cells. Live bacteria were isolated after 24 hours of oral or nasal immunization from mesenteric lymph nodes or NALT respectively. Interestingly IL2-GFP secreting LGG were translocated in higher numbers than GFP secreting LGG. Confocal microscopic examination of orally immunized mice intestine showed LGG were taken up by M cells. Transmission electron microscopic analyses also showed bacterial uptake in intestines.

3.4. Mucosal immunization with recombinant LGG

For mucosal immunization, the oral and nasal routes were examined. C57BL/6 and Balb/c mice were used to study the total or antigen specific immune response after oral immunization while the nasal immunization was performed only on C57BL/6 mice. Feces (oral immunization) or BAL fluid (nasal immunization) and sera were collected on days 0, 10, 24, 35 and 80 to study the mucosal and systemic immune responses respectively (Figure 3.7).

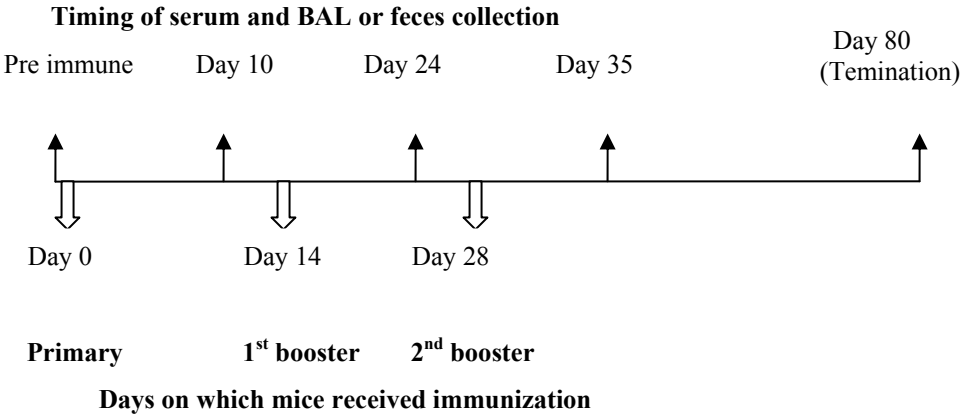


Figure 3.7. Oral or nasal immunization and sample collection schedule
Primary immunization begins on Day 0.

3.4.1. Systemic antibody production- general and specific after oral immunization

There was no significant increase of total serum IgG or IgA in either mice strain. As shown in Figure 3.8 (a - d). From the 24th day onwards, GFP specific IgG levels were significantly increased in both C57BL/6 and Balb/c mice immunized with modified LGG and on 35th day the IgG titres were significantly higher in LGG-IL-2-GFP immunized mice compared to the other groups (Figure 3.8 e&f).

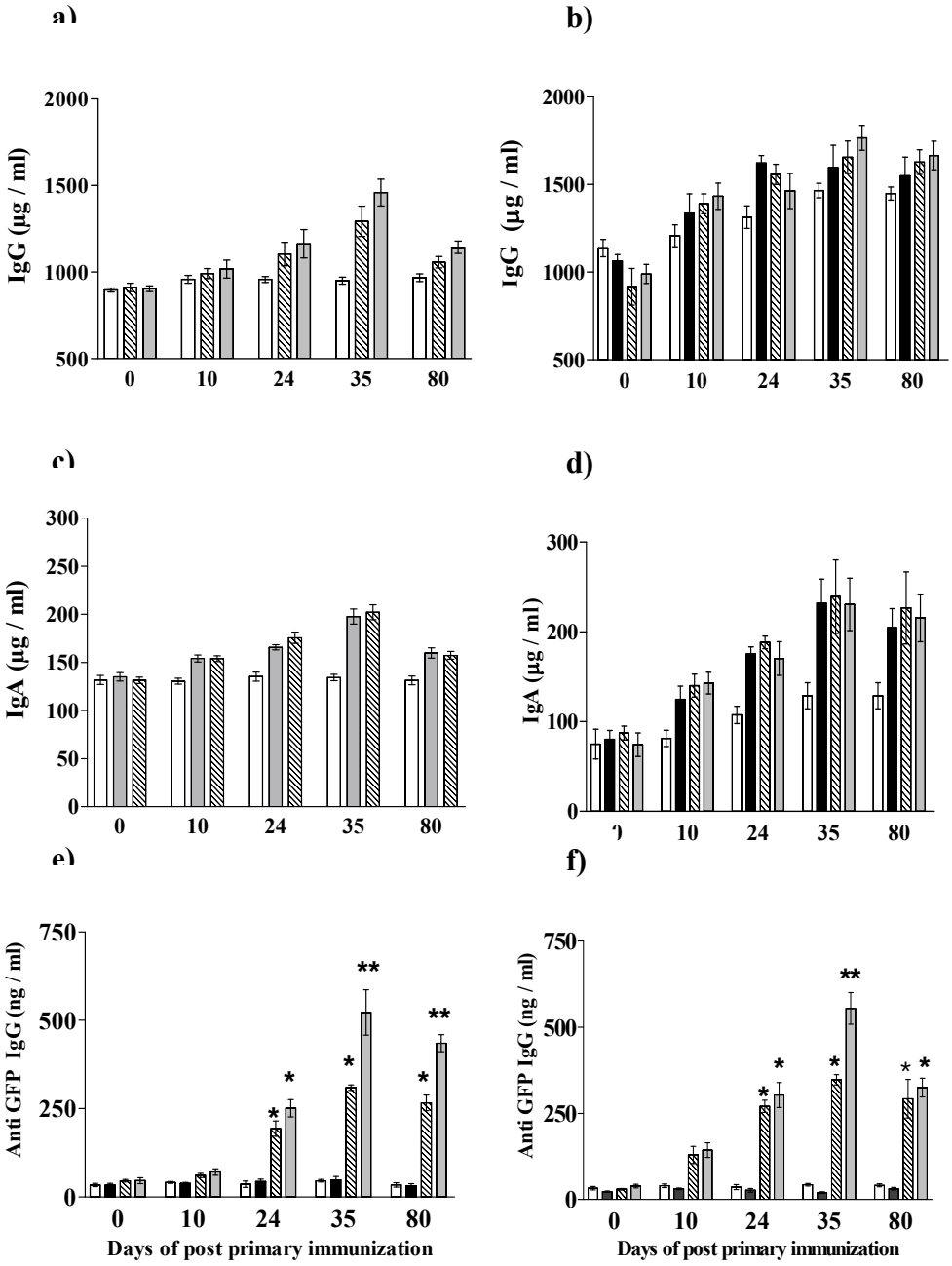


Figure 3.8. Total and GFP specific systemic immune induction in oral immunization.

Total and GFP specific serum IgG or IgA in C57BL/6 and Balb/c mice after oral immunization with modified LGG, wild type LGG and PBS. Eight mice from each group were inoculated on days 0, 14 and 28 with 10^8 modified LGG-GFP (angled stripes) or LGG-IL2-GFP (shaded) or wild type LGG (black) or with PBS (clear). Graphs a, c and e) represent total IgG, IgA and GFP pseciifc IgG in the sera of C57bL/6 mice. Graphs b,d and f) represent total IgG, IgA and GFP specific IgG from Balb/c mice.

*denotes statistical significance from PBS and LGG and ** indicates statistical significance from PBS, LGG and LGG-GFP ($p < 0.05$). Data are represented by the mean ± SEM in each group.

3.4.1.1. Local antibody production- general and specific

Secretory IgA (sIgA) levels increased significantly from day 10 and day 24 onwards in C57BL/6 and Balb/c mice respectively after immunization with LGG (Figure 3.9 a & b). The IgA antibody titers remained elevated 52 days after the last booster dose (day 80). GFP specific sIgA was also increased in fecal extracts from 24th or 10th day onwards in C57BL6 or Balb/c mice respectively (Figure.3.9 c&d). By the 35th and 80th days in the C57BL/6 mice the titre was significantly higher in mice immunized with LGG-IL2-GFP compared to the other groups.

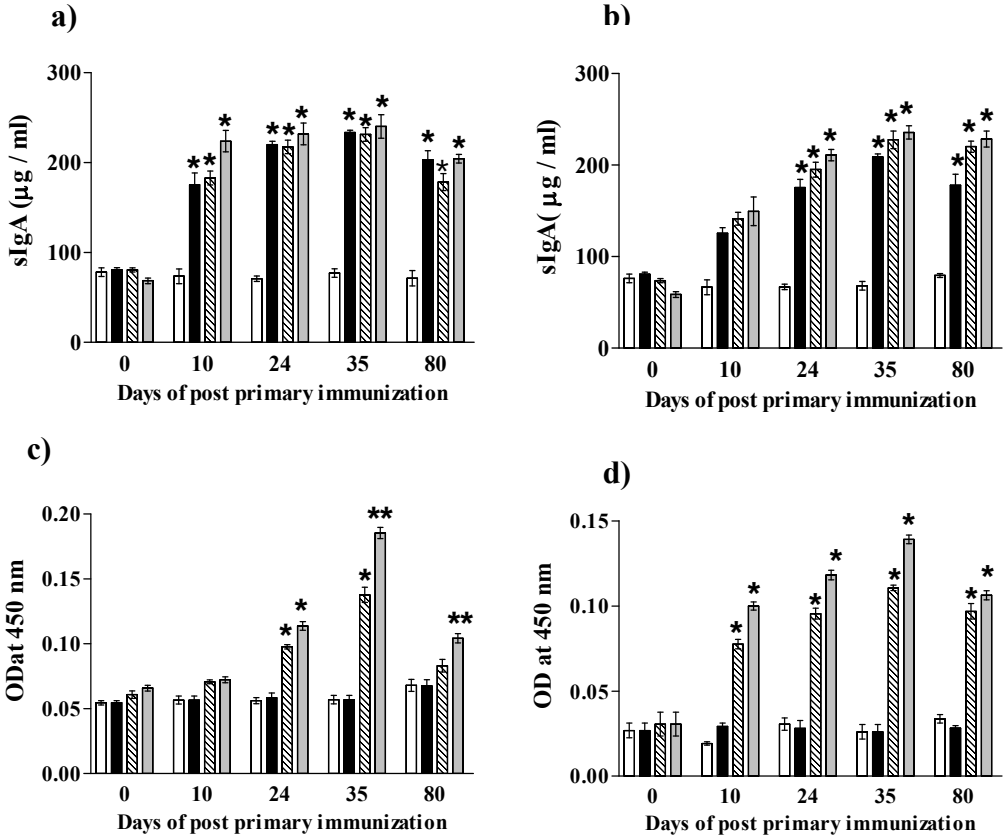


Figure 3.9. Total and GFP specific mouse IgA in fecal extracts of C57BL/6 or Balb/c mice after oral immunization with modified LGG, wild type LGG and PBS. a&b) Total mouse IgA in fecal extracts of C57BL/6 or Balb/c mice respectively.

c&d) GFP specific IgA in C57BL/6 and Balb/c mice after oral immunization. LGG-GFP (angled stripes) or LGG-IL2-GFP (shaded) or wild type LGG (black) or with PBS (clear). * denotes statistical significance from PBS and LGG and

** indicates statistical significance from PBS, LGG and LGG-GFP ($p < 0.05$). Data are represented by the mean ± SEM in each group.

3.4.1.2. IL2 co-expression enhanced GFP specific Ig production

The fecal extracts collected on 35th day were serially diluted to obtain approximately the same absorbance for total IgA/IgG and GFP IgA/IgG on the ELISA. The dilutions producing the same absorbance for GFP and total IgA/IgG were expressed as a percentage of the dilution of GFP over the dilution of total IgA/IgG. Similarly, the dilutions producing the same absorbance for GFP and LGG were used to determine the relative amount of GFP to LGG specific antibodies (fold difference).

Immunization with LGG-IL2-GFP and LGG-GFP resulted in 25 and 10 fold more GFP specific IgG and 5 and 2 fold more GFP specific IgA production in C57BL/6 mice whereas in Balb/c the ratios were lower primarily due to a higher production of LGG specific IgA after immunization (Figure 3.10).

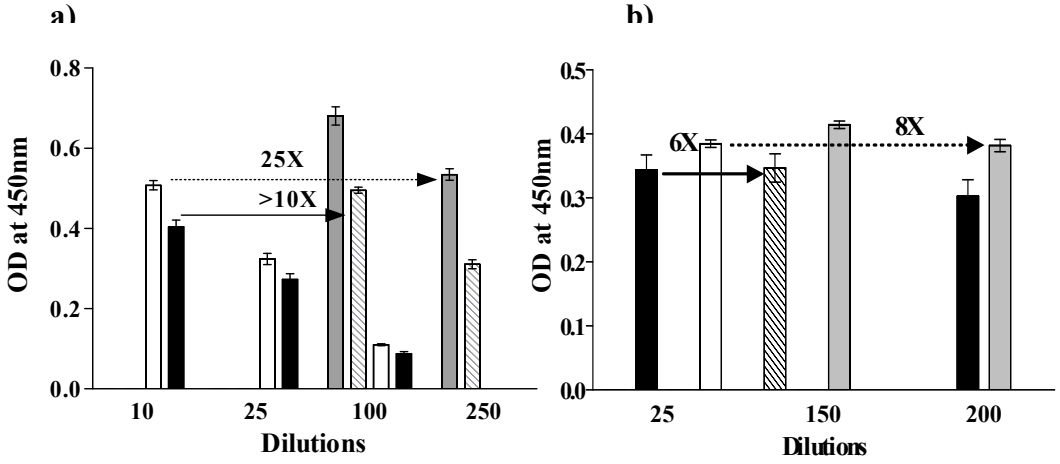


Figure 3.10. IL2 co-expression enhances GFP specific IgG induction.
At the 35th day of post primary immunization sera were analyzed for GFP or LGG specific IgG. a). Fold difference of GFP Vs LGG specific serum IgG induction in C57BL/6 mice. b) Fold difference of GFP Vs LGG specific IgG in Balb/c mice. Anti-GFP IgG (angled stripes) and anti-LGG IgG (black) in mice inoculated with LGG-GFP and anti-GFP IgG (shaded) and anti-LGG IgG (clear) in LGG-IL2-GFP inoculated mice. The difference in GFP and

Lactobacillus specific antibody levels were determined by comparing the dilutions of each required to obtain the same OD at 450 nm.

The mean percentage of GFP specific IgG (GFP specific IgG/Total IgG X 100) in LGG-IL2-GFP immunized mice was about 0.036 and 0.037 compared to 0.023 and 0.021 in LGG-GFP immunized C57BL/6 and Balb/c mice respectively. The percentage of GFP specific IgA was also higher in IL2-GFP-LGG immunized mice and was 0.063 and 0.050 compared to 0.011 or 0.012 in LGG-GFP immunized C57BL/6 and Balb/c mice respectively (Table. 3.1).

3.4.1.3. Analysis of GFP specific IgA and cytokines in intestinal fragment cultures

At day 35 the small and large intestine were harvested and 100 mg of tissue fragments were placed in culture media. After 24 hours, the culture supernatant was analyzed for total IgA, GFP specific IgA, IL2, IL6, IL4, TNFα, IFNγ and $TGF\beta_1$. Total IgA production was increased in both wild type and modified LGG immunized mice and it was significantly greater in the large intestine of LGG-IL2-GFP immunized mice than in PBS or LGG immunized mice respectively (Table. 3.2). Cultures of the small intestine, from LGG-IL2-GFP immunized mice produced a higher percentage of GFP specific IgA (0.063) compared to LGG-GFP (0.042) immunized C57BL6 mice. Similarly in the large intestines the ratio of GFP specific IgA was 0.02 for LGG-IL2-GFP immunized mice compared to 0.013 for LGG-GFP immunized C57BL6 mice.

There was no detectable IL2 or IFNγ. In LGG-IL2-GFP immunized mice small intestine culture, IL6 production was significantly higher and by contrast,

$TGF\beta_1$ levels were significantly lower when compared to PBS or LGG immunized mice (Table.3.2).

Table 3.3. Cytokine and IgA levels in intestinal culture supernatant

Group	TNFα (pg/ml)		IL6 (ng/ml)		sIgA (μg/100 g of tissue)		TGFβ (ng/ml)	
	Small intestine	Large intestine	Small intestine	Large intestine	Small intestine	Large intestine	Small intestine	Large intestine
PBS	9.3 ± 3.6	6.1 ± 1.9	0.09 ± 0.06	0.53 ± 0.14	15.3 ± 2.2	0.8 ± 0.1	7.78 ± 2.50	2.63 ± 0.21
LGG	4.2 ± 3.7	14.2 ± 4.1	0.12 ± 0.07	0.90 ± 0.15	31.1 ± 6.7	2.1 ± 1.0	6.21 ± 3.02	2.84 ± 0.20
LGG-GFP	11.1 ± 1.1	10.7 ± 5.1	0.23 ± 0.13	0.91 ± 0.39	35.0 ± 4.1	8.6 ± 4.8	6.02 ± 2.97	1.84 ± 0.28♦
LGG-IL2-GFP	9.9 ± 6.8	20.1 ± 10.6	0.63 ± 0.30*	0.99 ± 0.31	38.0 ± 6.8	12.8 ± 7.0*	1.65 ± 1.07*	3.39 ± 0.69

Data represent mean % of cells ± standard deviations

* Statistically significant ($p < 0.05$) compared to PBS and LGG administration

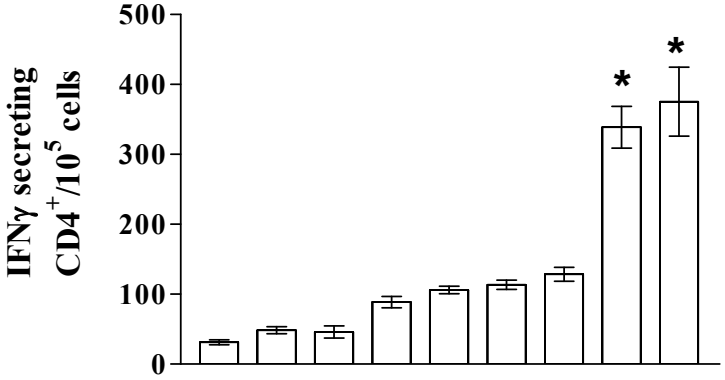
♦ Statistically significant ($p < 0.05$) compared to LGG and LGG-IL2-GFP administration

3.4.1.4. IFNγ ELISPOT for antigen specific CD4 and CD8 T cell responses

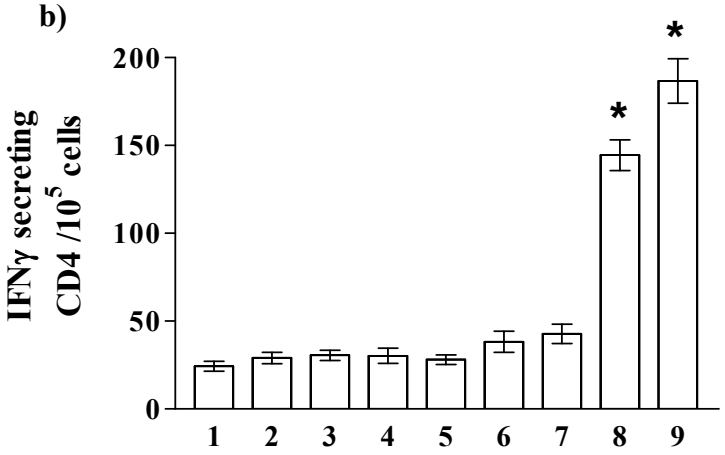
To monitor the induction of CD4 and CD8 antigen specific T cells, an IFN-γ Elispot assay was performed on splenocytes isolated on days 35 and 80. Increased IFNγ secreting $CD4^+$ or $CD8^+$ T cells were seen after modified LGG immunization

($p < 0.05$) in both C57BL6 and Balb/c mice. LGG-IL2-GFP immunization generated significantly higher GFP specific $CD8^+$ T cells (187 ± 31) than LGG-GFP (90 ± 25) in C57BL6 mice at day 35. A similar response, 121 ± 23 and 69 ± 11 $CD8^+$T cells from LGG-IL-2-GFP and LGG-GFP immunized mice was also observed in Balb/c mice at day 80 (Figure. 3.11).

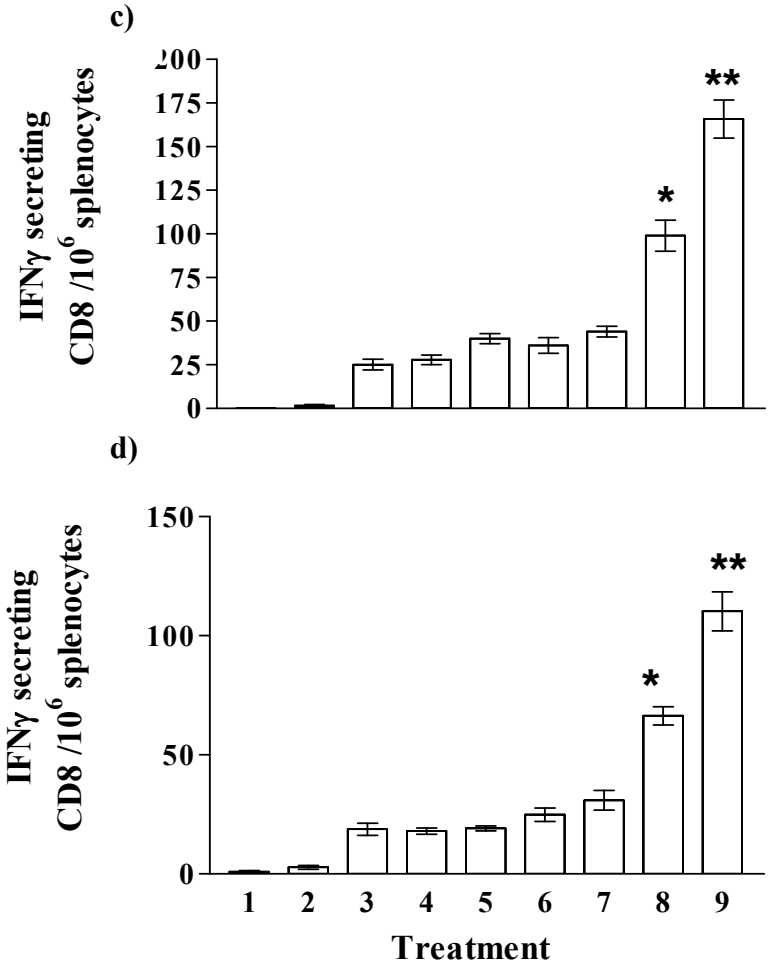
a)



b)



Mit C treated splenocytes	+	+	+	+	+	+	+	+	+
rGFP	-	-	-	-	-	+	+	+	+
CD4+T from PBS	-	+	-	-	-	+	-	-	-
CD4+T from LGG	-	-	+	-	-	-	+	-	-
CD4+T from LGG-GFP	-	-	-	+	-	-	-	+	-
CD4+T from LGG-GFP-IL2	-	-	-	-	+	-	-	-	+



Mit C treated splenocytes	+	+	+	+	+	+	+	+	+
GFP CTL peptide	-	-	-	-	-	+	+	+	+
Splenocytes from PBS	-	+	-	-	-	+	-	-	-
Splenocytes from LGG	-	-	+	-	-	-	+	-	-
Splenocytes from LGG-GFP	-	-	-	+	-	-	-	+	-
Splenocytes from LGG-GFP-IL2	-	-	-	-	+	-	-	-	+

Figure 3.11. IFN-γ ELISPOT for the analysis of GFP specific $CD4^+$ or $CD8^+$ T cells More expln here

GFP specific $CD4^+$T or $CD8^+$ T cell responses in C57BL6 (a&c) or Balb/c (b&d) mice. * indicates statistical significance from PBS and LGG immunized mice and ** indicates statistical significance from PBS, LGG and LGG-GFP ($p < 0.05$). Data are represented as the mean ± SEM in each group.

3.4.1.5. Immunization with LGG-GFP and IL2-GFP-LGG produced a GFP specific CTL response

A CTL assay was performed on splenocytes from immunized mice at day 35. Splenocytes were stimulated *in vitro* with mitomycin C treated MB49-GFP cells and GFP specific CTL peptide. Stimulated splenocytes were then tested for recognition and lysis of fresh viable MB49-GFP cells. As shown in figure.3.12, spleen cells from mice immunized with LGG-IL2-GFP or LGG-GFP had significant CTL activity compared to LGG or PBS immunized mice. LGG-IL2-GFP immunized mice produced significantly more CTL activity than LGG-GFP immunized mice at all effector to target cell ratios.

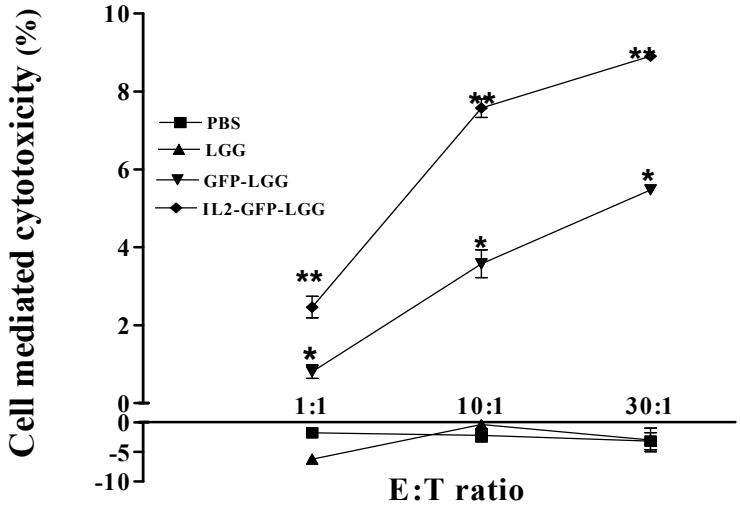


Figure 3.12. Induction of GFP specific CTL response in splenocytes of mice immunized with recombinant LGG.
Four mice per group immunized via the oral route on days of 0, 14 and 28 with either PBS or wild type LGG or LGG-GFP or LGG-IL2-GFP were killed on day 35. Splenocytes from immunized mice were pooled and co-cultured with mitomycin C treated MB49-GFP cells and CTL peptide of GFP for 5 days. These splenocytes were used as effector cells and live MB49-GFP cells served as target cells. The assay was performed at the following effector to target (E:T) ratios (1:1,10:1,and 30:1). Specific lysis was determined by quantitative measurements of LDH.
* indicates statistical significance from PBS and LGG immunized mice and
** indicates statistical significance from PBS, LGG and LGG-GFP ($p < 0.05$). Data are represented as the mean ± SEM in each group.

3.4.1.6. Phenotyping of mononuclear cell subsets in MLN after oral immunization

Single cell suspensions from the MLN were prepared from the mice on the 35th day after primary immunization. Both wild type and modified LGG immunization increased the number of $CD4^+$, $CD8^+$ T cells and IgA^+B cells in MLN compared to mice receiving PBS. LGG-IL2-GFP immunization induced increased maturation of DC at MLN (Table. 3.3).

Table 3.4. Mononuclear cell subsets in MLN on the 35th day after immunization

IImmunization	$B220^+$ IgA^+	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD11c^+CD86^+$	$CD11c^+CD80^+$
PBS	12.1 ± 2.15	17.7 ± 2.84	20.6 ± 1.64	73.7 ± 3.14	25.9 ± 3.26
LGG	18.7 ± 4.47	35.9 ± 4.34*	38.6 ± 4.50*	78.7 ± 4.41	28.3 ± 2.94
LGG-GFP	18.1 ± 2.90	35.2 ± 3.48*	38.8 ± 4.60*	82.3 ± 4.57	28.3 ± 5.77
LGG-IL2-GFP	20.0 ± 2.67*	36.8 ± 3.30*	38.0 ± 2.22*	86.0 ± 7.04*	30.1 ± 3.81

Data represent mean % of cells ± standard deviations
* Statistically significant ($p < 0.05$) compared to PBS.

Summary II

In this present study both recombinant LGG were shown to be able to elicit mucosal and systemic humoral responses after oral immunization in both C57BL/6 and Balb/c mice. Oral immunization of LGG elicited a sustained mucosal IgA induction and *ex vivo* culture of the intestines also showed a higher IgA level even 56 days after the last booster immunization. *Lactobacillus* delivery of GFP (approximately 0.1 ng) resulted in approximately 0.3 μg/ml of GFP specific IgG antibodies being produced from LGG-GFP, while co-delivery of IL2 and GFP resulted in more antibody production namely 0.5 μg/ml of GFP specific antibodies. Our data indicates that a low level of secreted IL2 (approximately 20 pg) is sufficient to produce significant adjuvant effects a 1.6 fold increase in antibody production. The increased level of anti-LGG antibodies was not as great as the increase in GFP antibodies produced by the IL-2 fusion protein. Thus Lactobacilli are excellent delivery vehicles as they do not evoke a strong antibody response against themselves. There were increased GFP specific $CD8^{+}$T cells in the recall assay and these were increased by IL2 co-expression. The CD8 T cells were cytotoxic to MB49 cells secreting GFP. The results of this study showed the beneficial effect of IL2 expression as a fusion protein with an antigen to elicit enhanced local and systemic humoral immune response as well as cellular immune response.

3.4.2. Nasal Immunization.

Previous results with *Lactobacillus plantarum* expressing human papilloma virus 16 (HPV-16) E7 antigen demonstrated that intranasal route immunization was more effective than intragastric immunization (Cortes-Perez *et al.* 2007). As the choice of route of immunization is a crucial step in developing live mucosal vaccines, the immune induction elicited by nasal immunization was compared with the results obtained in oral immunization.

3.4.2.1. General and specific antibody induction in nasal immunization

Nasal immunization results in brisk and sustained induction of serum IgM, IgA, IgG and mucosal IgA (Waldo *et al*, 1994). Moreover intranasal co-administration of lactobacilli with cow's milk allergen prevented the mice from sensitization (Cortes-Perez *et al*, 2007). Hence serum IgM, IgG, IgA and IgE were measured after nasal immunization. The serum IgA levels (Figure 3.13 a) increased after the priming dose and were significantly increased by day 35 (LGG-IL2-GFP) and remained high at day 80 (both modified microbes). There was a slight increase in total serum IgG (Figure 3.13 b) and IgM antibodies (data was not shown) but it was not statistically different from PBS or LGG administered mice and there was no change in IgE levels (data was not shown).

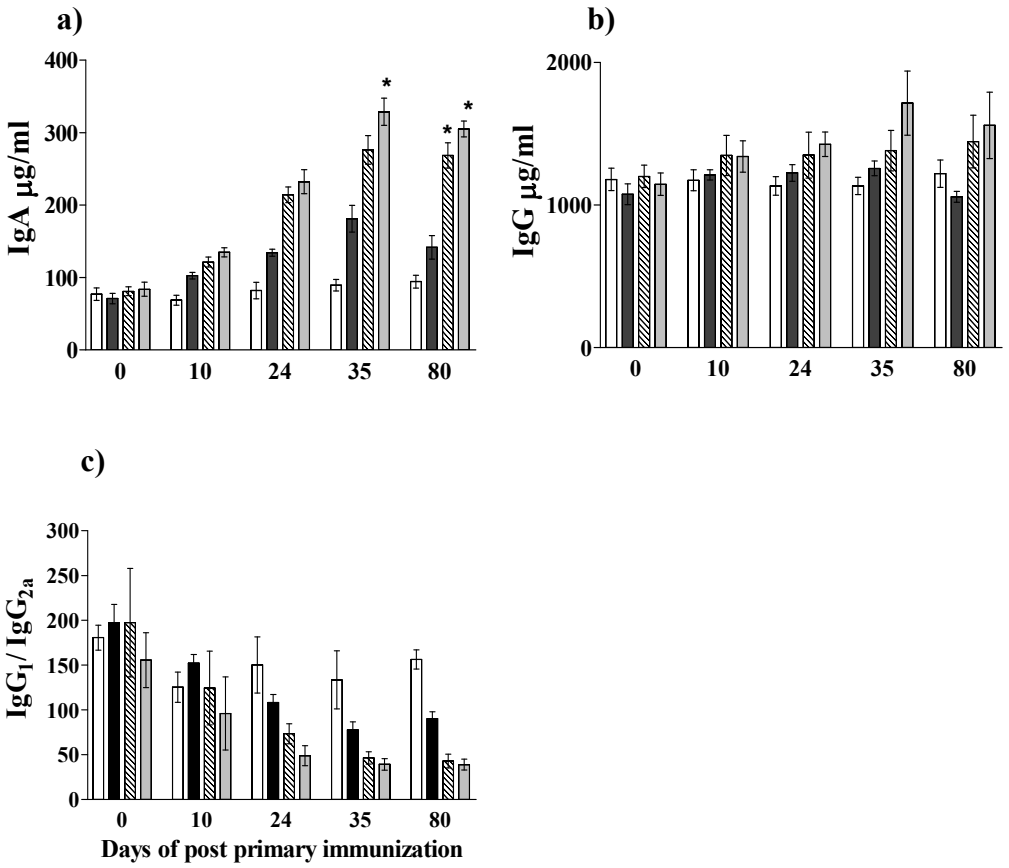


Figure 3.13. Systemic immune response in C57BL/6 mice after nasal immunization with of PBS or LGG or LGG-GFP or LGG-IL2-GFP. a, b, represent the mouse serum IgA, IgG induced after nasal immunization with LGG-GFP (angled stripes) or LGG-IL2-GFP (shaded) or wild type LGG (black) or PBS (clear) and c) represents the serum $IgG_1/IgG2_a$ ratio.
* denotes statistically significant with PBS or LGG immunization. ($p < 0.05$). Data are represented as the mean ± SEM in each group.

GFP specific IgG titers increased rapidly (Figure 3.14), IL2 significantly increased GFP specific IgG production. The GFP specific IgG_1/IgG_{2a} ratio initially increased due to increasing IgG_1 levels and decreased later as IgG_{2a} levels increased (day 35 maximal, Fig. 3.14.b). GFP specific IgA in serum increased rapidly and with similar kinetics and profile as total IgA. Anti-GFP IgA levels were significantly increased in mice immunized with LGG-IL2-GFP compared to the other groups as seen in Figure 3.14.c. Similarly, anti-GFP sIgA levels were differentially induced by modified LGG in the BAL (Fig 3.14.e).

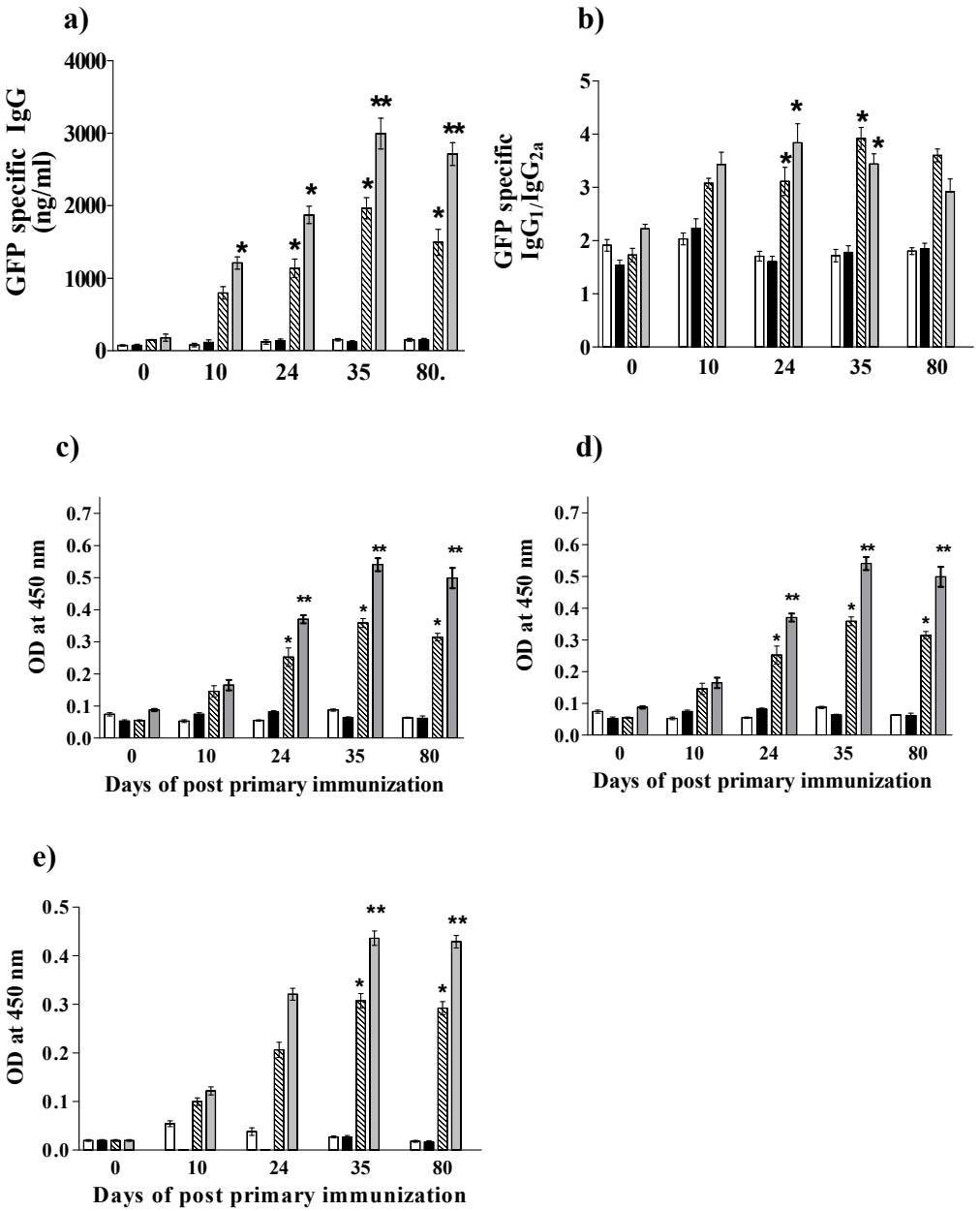


Figure 3.14. GFP specific local or systemic immune response induced in serum or BAL in C57BL/6 mice after nasal immunization with of LGG or modified LGG.

a) GFP specific IgG b) GFP specific IgG1/IgG2a c) GFP specific IgA induced in serum. d) Total sIgA and e) GFP specific IgA induced in BAL. LGG-GFP (angled stripes) or LGG-IL2-GFP (shaded) or wild type LGG (black) or PBS (clear)

* denotes statistically significant with PBS or LGG immunization and

** denotes statistically significant with PBS or LGG or LGG-GFP immunization. ($p < 0.05$). Data are represented as the mean ± SEM in each group.

3.4.2.2. Immune induction at ectopic mucosal tissues

Nasal immunization also induced IgA secretion in other mucosal sites as well. IgA secreted in intestines particularly from LGG-IL2-GFP immunized mice were statistically significant with PBS immunized mice as seen in Fig3.15.a. Though the OD for GFP specific IgA in modified LGG immunized mice vaginal mucosa was very low, it was statistically significant with wild type LGG immunized mice (Fig 3.15.b).

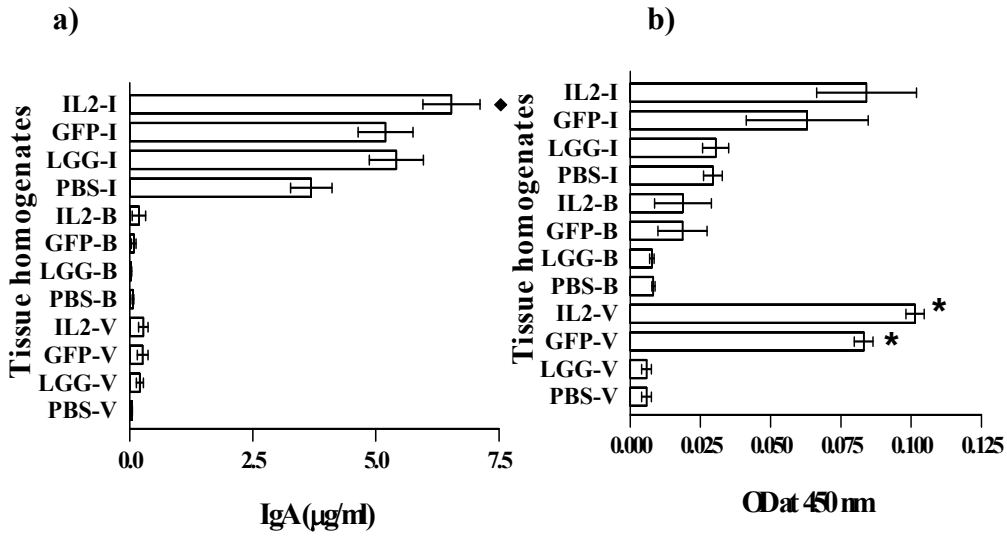


Figure 3.15. Total and GFP specific IgA produced at the intestinal(I), bladder(B), and Vaginal mucosa (V) at the 80th day after nasal immunization. a) Total mouse IgA and b) GFP specific IgA. Tissues. * denotes statistical significance from PBS and LGG and ♦ denotes statistical significance from PBS, $p<0.05$. Data are represented by the mean ± SEM in each group.

3.4.2.3. Antibody induction by intranasal immunization with LGG-IL2-GFP was more antigen specific

At day 35, immunization with LGG-IL2-GFP and LGG-GFP induced approximately 32 and 24 times more GFP than LGG specific IgG or 12 and 10 fold more anti-GFP IgA relative to anti-LGG IgA antibodies in serum or BAL respectively (Fig 3.16).

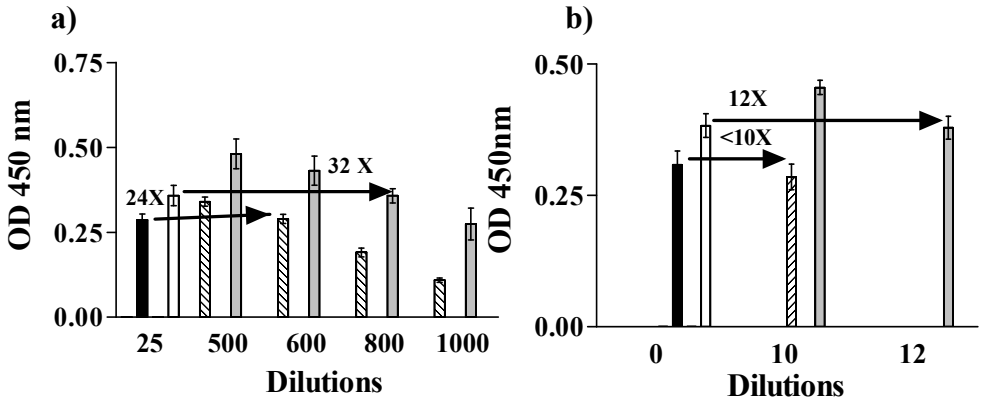


Figure 3.16. Analysis of GFP Vs LGG specific antibody induction
At the 35th day post primary immunization, serum or BAL were analyzed for GFP or LGG specific IgG or IgA respectively. a). Fold difference of GFP Vs LGG specific serum IgG induction in serum. b) Fold difference of GFP Vs LGG specific IgA in BAL. Anti-GFP IgG or IgA (angled stripes) and anti-LGG IgG or IgA (black) in mice inoculated with LGG-GFP and anti-GFP IgG or IgA (shaded) and anti-LGG IgG or IgA(clear) in LGG-IL2-GFP inoculated mice. The difference in GFP and *Lactobacillus* specific antibody levels were determined by comparing the dilutions of each required to obtain the same OD at 450 nm.

3.4.2.4. Analysis of total and GFP specific IgA in CLN, NALT and lung tissue *ex vivo*

At the 35th day post primary nasal immunization, the CLN, NALT and lung tissues from immunized mice were harvested and cultured *ex vivo* for 24 hours. CLN and lung cells in LGG immunized mice produced a high level of IgA or

IgG (Fig 3.17). In LGG-IL2-GFP immunized mice, lung and CLN cells produced a higher GFP specific IgA or IgG which was statistically significant from LGG immunized mice as seen in figure 3.17. c&d.

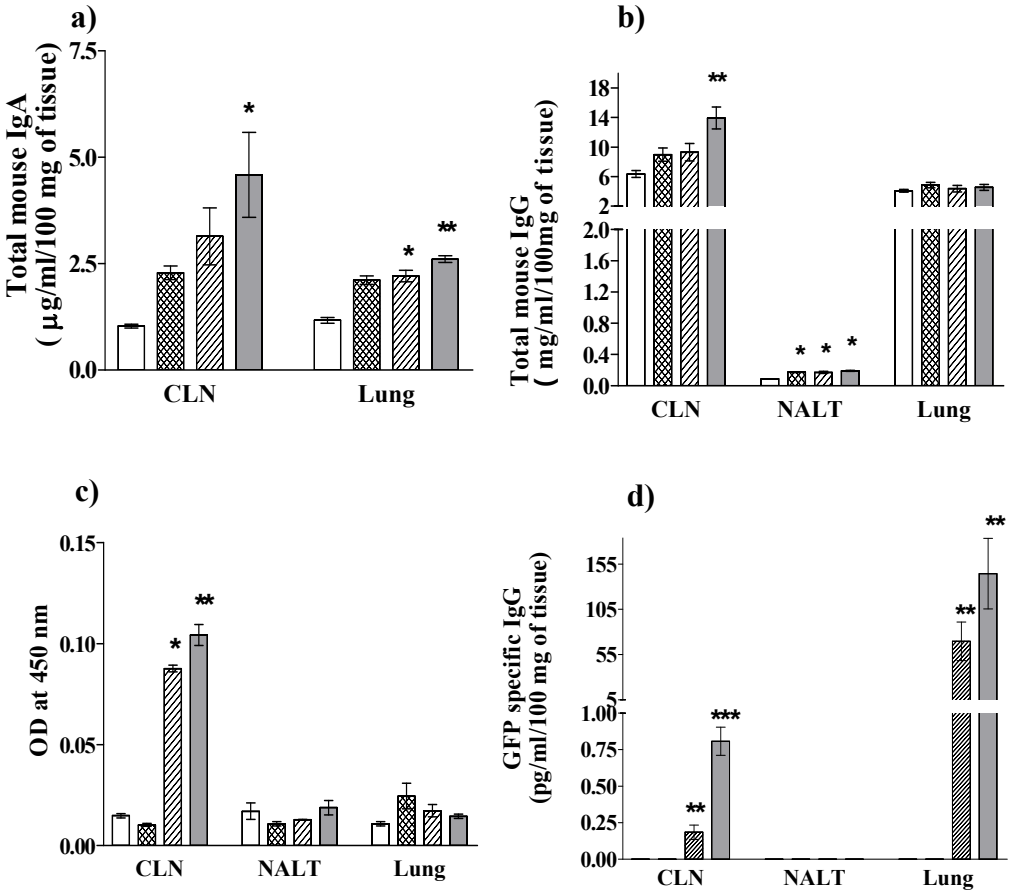


Figure 3.17. Total or GFP specific Ig induction in *ex vivo* culture.

At the 35th day post primary immunization, CLN, NALT and lung tissues of the mice immunized with LGG-GFP (angled stripes) or LGG-IL2-GFP (shaded) or wild type LGG (crossed stripes) or PBS (clear) were aseptically harvested and cultured ex vivo for 24 hours and supernatants were analyzed for total (a & b) or GFP specific IgA or IgG (c & d). For lung and CLN, 100mg of tissues were cultured and for NALT, the whole tissue was cultured. * denotes statistical significance from PBS, ** denotes statistical significance from PBS and LGG and *** denotes statistical significance from PBS, LGG, LGG-GFP, $p < 0.05$. Data are represented by the mean ± SEM in each group.

3.4.2.5. Analysis of inflammatory cells in BAL after nasal immunization.

LGG-IL-2-GFP immunized mice had significantly more lymphocytes in the BAL than LGG immunized mice (Fig 3.18e). Macrophage and neutrophil numbers did not differ between the different LGG groups. By the 80th day, the pattern of immune cells was almost back to that in PBS treated mice except for lymphocytes which, though much lower than at day 35, were significantly higher in mice immunized with modified LGG (Fig 3.18f).

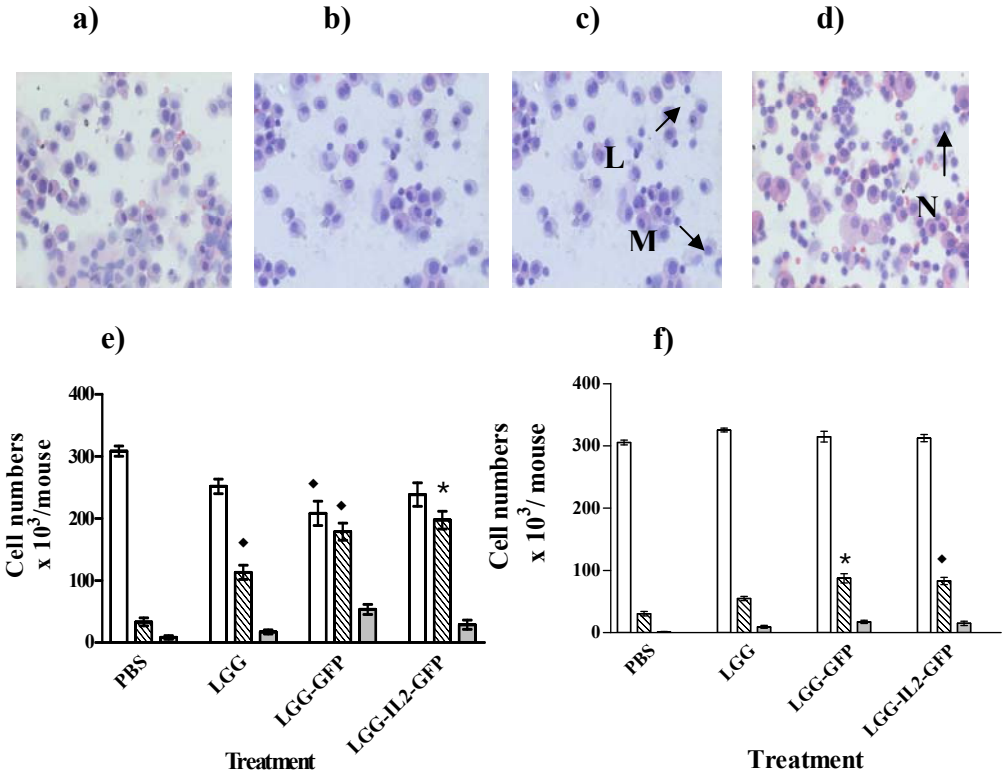


Figure 3.18. Immune cells in BAL fluid after immunization with modified LGG or wild type LGG or PBS. On days 35 and 80, BAL fluid cells from immunized mice were cytospined and H & E stained. Cells were visualized and examined under microscopy (magnification 2 x 20 - a to d). M - monocytes, L - lymphocytes, N- neutrophils. The number of macrophages/monocytes (clear), lymphocytes (striped) and neutrophils (shaded) in 5000 cells were enumerated. Immune cell populations in e) day 35 and f) day 80 BAL fluid samples are shown. * indicates statistical significance from PBS and LGG administration and ♦ indicates statistical significance from PBS administration. Data are represented as the mean ± SEM in each group.

3.4.2.6. Cytokine levels in BAL on the 35th day after nasal immunization

At day 35 only IFN-γ was significantly increased in the BAL of mice administered LGG-IL2-GFP compared to mice given LGG-GFP, LGG and PBS (Table 3.5). While TNFα was significantly greater in the BAL of both modified LGG compared to LGG and PBS administered mice. IL12, IL4 and IL10 levels in BAL were similar in all groups.

Table 3.5 . Cytokine levels in fluid from lung lavage on the 35 th day after nasal immunization

Immunization	Cytokine levels in lung lavage fluid (pg/ml)					
	TNFα	IL12	IFNγ	IL6	IL10	IL4
PBS	68.0 ± 22.02	392.6 ± 179.3	74.15 ± 7.38	81.5 ± 26.07	158.5 ± 32.46	15.66 ± 7.78
LGG	107.74 ±41.93	501.49 ± 111.15	81.47 ± 32.49	142.6 ± 15.64	182.8 ± 28.89	11.41 ±4.23
LGG-GFP	158.48 ± 30.61 ♦	513.68 ± 142.88	105.89 ± 31.47	137.5 ± 19.03	172.0 ± 17.43	11.62 ± 4.15
LGG-IL2-GFP	186.05 ± 34.13 *	649.19 ± 71.13	177.87 ± 32.68 **	189.21 ± 19.95 ♦	177.3 ± 13.95	8.74 ± 5.88

This experiment was repeated twice and the data shown here is the mean ± standard deviation from one experiment.
** Statistically significant ($p < 0.05$) compared to PBS, LGG, LGG-GFP administration
*Statistically significant ($p < 0.05$) compared to PBS, LGG administration
♦ Statistically significant ($p < 0.05$) compared to PBS administration

3.4.2.7. Phenotyping of cells in CLN and NALT after intranasal immunization

Single cell suspensions from the CLN and NALT were prepared from the mice on days 10, 24 and 35. Though the differences are small, the modified LGG immunization elicited significantly higher numbers of IgA^+ B cells and $CD8^+$ T cells in CLN. A Significant difference in IgA^+ B cell numbers was only observed at day 35 in NALT with LGG-IL2-GFP imunization (Table 3.6). While LGG administration generally increased the number of $CD4^+$ cells.

Table 3.6. Mononuclear cell subsets in CLN and NALT on the 35th day after immunization

Immunization	CLN x10^6 cells				NALT % of cells
	B220 IgA^+	$CD3^+CD4^+$	$CD3^+CD8^+$	Mac^+	B220 IgA^+
PBS	1.38 ± 0.13	1.17 ± 0.11	1.61 ± 0.11	1.00 ± 0.09	13.6 ± 4.12
LGG	2.20 ± 0.14	1.87 ± 0.12♦	2.14 ± 0.14	1.88 ± 0.12♦	22.6 ± 5.16
LGG-GFP	3.03± .26*	2.00 ± 0.17♦	2.59 ± 0.22*	1.38 ± 0.12♦	40.0 ± 13.66
LGG-IL2-GFP	3.53± .36*	2.06 ± 0.21♦	2.58 ± 0.26*	0.91 ± 0.05	56.3 ± 1.29*

Data represent mean absolute number or % of cells ± standard deviations
* Statistically significant ($p < 0.05$) compared to PBS and LGG administration
♦ Statistically significant ($p < 0.05$) compared to PBS administration

3.4.2.8. Histopathological analyses and immunohistochemical staining of the lungs from immunized mice

As the lung are not the usual site for LGG, it was necessary to determine if nasal immunization with LGG would lead to tissue damage. Lung tissues were examined for signs of immune cell accumulation and fibrosis. At the 35th day (Fig 3.19.a) there was increased cellular infiltration in the peribronchiolar regions of the lung tissues, isolated from LGG, LGG-GFP and LGG- IL2-GFP immunized mice compared to PBS immunized mice. However the cellular infiltrates were reduced by day 80 (Fig 3.19.b). Immunohistochemical staining of lungs from immunized mice showed the nasal immunization of wild type LGG or modified LGG induced IgA secretion from B cells (Figure. 3.19.c).

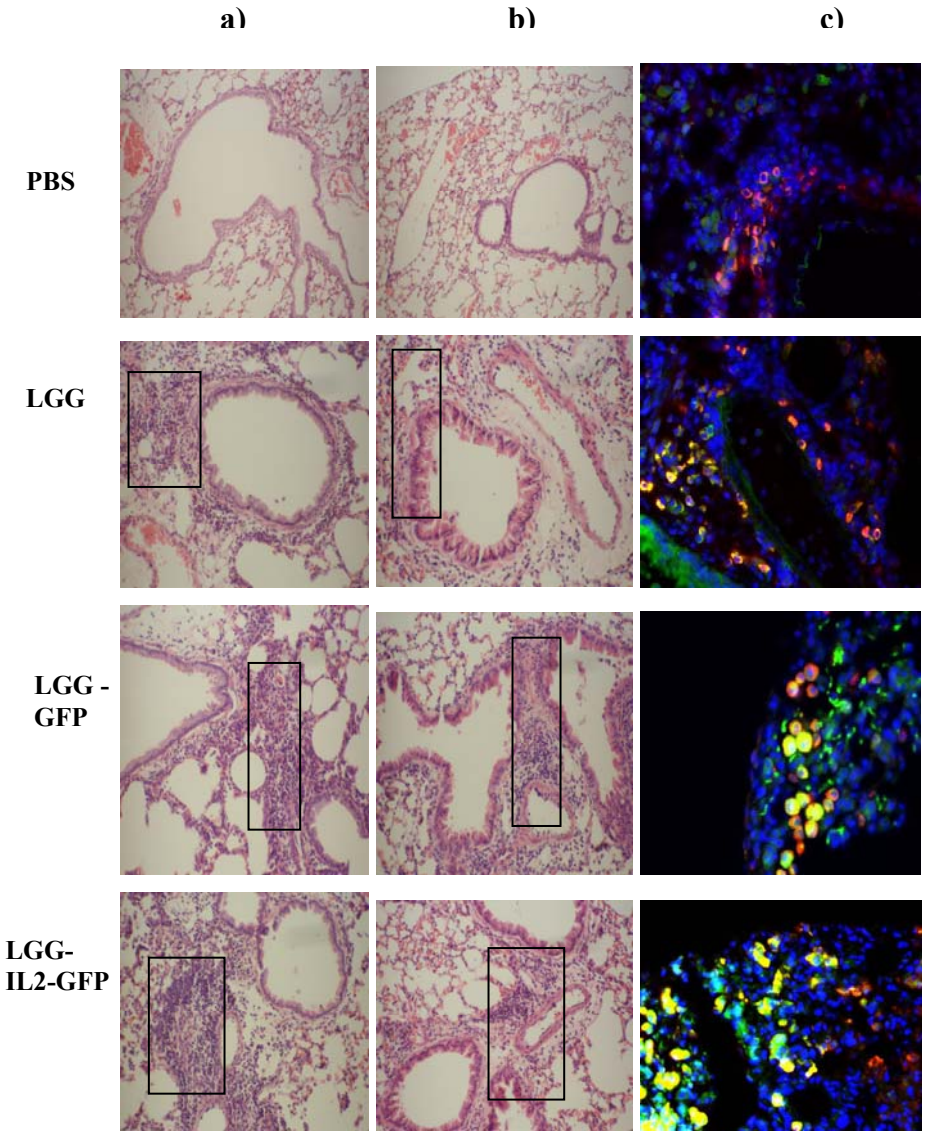


Figure 3.19. Histopathological analyses and immunohistochemical staining of IgA secreting B cells in murine lung tissue.
On days 35 and 80, lungs were harvested from immunized mice and fixed in 10% formalin. The paraffin embedded tissue sections were H & E stained and evaluated for cellular infiltration or inflammatory changes (boxed region). Tissue sections of the mice immunized with PBS , LGG , LGG-GFP and LGG-IL2-GFP which were harvested on the 35^{th} (a) and 80^{th} (b) days. On 80^{th} day lungs from mice immunized with PBS or LGG or LGG-GFP or LGG-IL2-GFP were cryopreserved and 5-10µm size frozen sections were stained with rhodamine labeled anti mouse IgA and FITC labeled anti mouse B220 antibodies followed by counter staining with DAPI. Sections were examined under confocal microscopy (c). Yellow fluorescent $B220^{+}$ IgA^{+} cells were easily distinguished from red fluorescent IgA^{+} or green fluorescent $B220^{+}$ cells. (magnification 2 x 20)

3.4.2.9. Analysis of mouse inflammatory cytokines and receptors with microarray in lungs of immunized mice

One lung was harvested from each group at the 35th day post primary immunization to study the mRNA profile of inflammatory cytokines, chemokines and their receptors. At 35 the day Ccl17,Ccl2, Ccl25, Ccl6, Ccr2, Fcgr1, Cxcl5, Il10, Il16, Il1r1, Il20, Il2rg, and Il5ra genes were differentially expressed in the lungs of the mice immunized with PBS or LGG or LGG-GFP or LGG-IL2-GFP (figure 3.20 and Table 3.7).

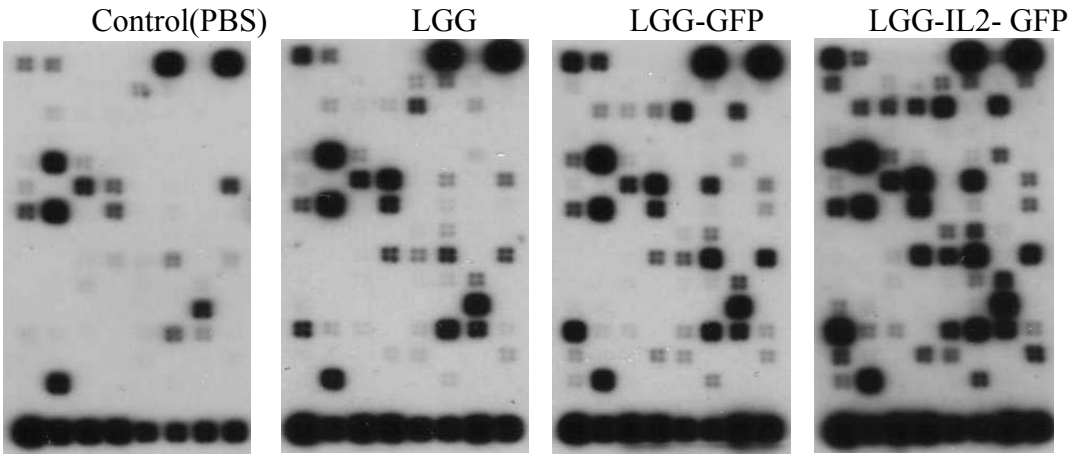


Figure 3.20 . X - Ray images of cRNA array – at 35th day- Gene expression profiling of the mouse lung harvested at 35th day of post primary immunization with PBS or LGG or LGG-GFP or LGG-IL2-GFP.

Table 3.7. List of the genes expression upregulated on oligo array.

	Symbol	**Fold increase on oligoarray at 35th day of post primary immunization**		
		LGG / control	LGG-GFP/ control	LGG-IL2-GFP / control
NM_011332	Ccl17	6.03	6.78	7.89
NM_011333	Ccl2	0.28	0.238	5.09
NM_009138	**Ccl25**	28.06	66.64	82.27
NM_009139	Ccl6	19.27	13.26	50.09
NM_009915	**Ccr2**	1.26	4.04	6.21
NM_010186	**Fcgr1**	8.83	2.24	9.39
NM_009141	Cxcl5	12.22	44.09	21.40
NM_010548	Il10	75.57	56.41	20.81
NM_010551	**Il16**	0.11	0.426	4.84
NM_008362	Il1r1	10.05	15.78	20.03
NM_021380	Il20	11.74	7.62	11.73
NM_013563	Il2rg	6.03	7.23	11.73
NM_008370	Il5ra	46.19	28.27	61.05

Ccl25, Ccr2, Fcgr1 and Il16 (bold and underlined) up regulation were confirmed with PCR. Apart from the genes screened in oligo array, IP-10 and CD8a genes were also analyzed with PCR.

At the 35th day post primary immunization IL16, CCR2, IP10, CD8a and CCL25 mRNA transcripts were significantly increased after LGG-IL2-GFP immunization. IL16 expression in LGG-IL2-GFP immunized mice lung was statistically significant with PBS immunization (Table 3.8). CCR2, IP10, CD8a or CCL25 expression in LGG-IL2-GFP immunized mice lung was statistically significant from PBS, LGG, LGG-GFP or PBS, LGG immunization respectively (Figure 3.21)

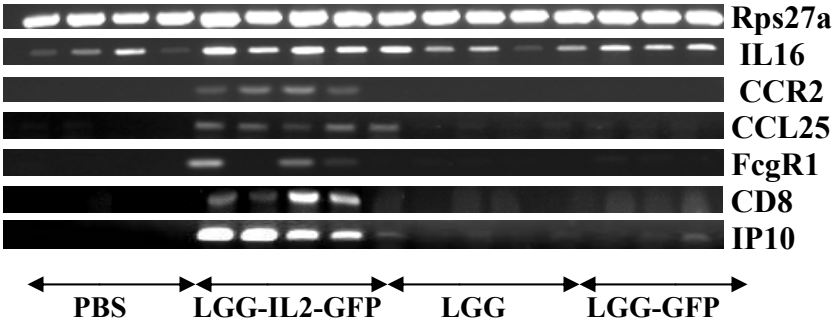


Figure 3.21. Gene expression changes induced by the recombinant or wild type LGG in lung.
To confirm the array data cDNA was synthesized from total RNA isolated from the lungs of PBS (n = 4), LGG (n = 4), LGG-GFP (n = 4) and LGG-IL2-GFP (n = 4) immunized mice on the 35th day post primary immunization. PCR was performed to confirm and identify gene expression changes. The following genes were found to be differentially expressed: IL16, CCR2, CCL25, IP-10, CD8a and FcgR1.

Table 3.8. Relative expression of chemokine genes analyzed by semi-quantitative PCR

Gene	Treatment			
	PBS	LGG	LGG-GFP	LGG-IL2-GFP
IL16	0.292 ± 0.280	0.656 ± 0.494	0.808 ± 0.244	1.144 ± 0.2 *
CCR2	-	-	0.014 ± 0.007	0.156 ± 0.05***
FcgR1	-	-	-	0.309 ± 0.36
CCL25	0.054 ± 0.064	0.061 ± 0.013	0.162 ± 0.093	0.427 ± 0.27**
IP10	0.085 ± 0.026	0.279 ± 0.029	0.166 ± 0.042	0.806 ± 0.16***
CD8a	-	-	0.005 ± 0.009	0.161 ± 0.12***

Data expressed is relative to Rps27a; '-' indicates not detectable. Data is presented as mean ± SD. * denotes statistically significant with PBS, ** denotes statistically significant with PBS and LGG and *** denotes statistically significant with PBS, LGG and LGG-GFP. P value < 0.05.
Gene expression was normalised with Rps27a levels. The PCR product band intensities were quantified with SynGene GeneTools analysis software. The values of the mRNA transcripts were normalized against Rps27a.

3.4.2.10. Induction of GFP specific cellular immune response by nasal immunization

Antigen specific cellular immune was analyzed by *ex vivo* ELISPOT assays for IFNγ secreting $CD4^+$ or $CD8^+$ T cells on splenocytes isolated on day 80 or 35 respectively. Increased IFNγ secreting $CD4^+$ (Figure 3.22.a) and $CD8^+$ T cells (Figure 3.22.b) were seen after modified LGG immunization ($p < 0.05$). LGG-GFP or LGG-IL2-GFP immunization generated about 314 ± 129 or 401 ± 109 GFP specific CD4+T cells respectively. LGG-IL2-GFP immunization generated significantly higher GFP specific $CD8^+$ T cells (197 ± 27) than LGG-GFP (105 ± 23). The cytotoxic activity of these cells against a GFP expressing cancer cell line, MB49-GFP was examined. The CTL activity in splenocytes isolated from mice immunized with LGG-IL2-GFP was about 2 fold greater than in those immunized with LGG-GFP, which was consistent with the Elispot data (Fig 3.22.c).

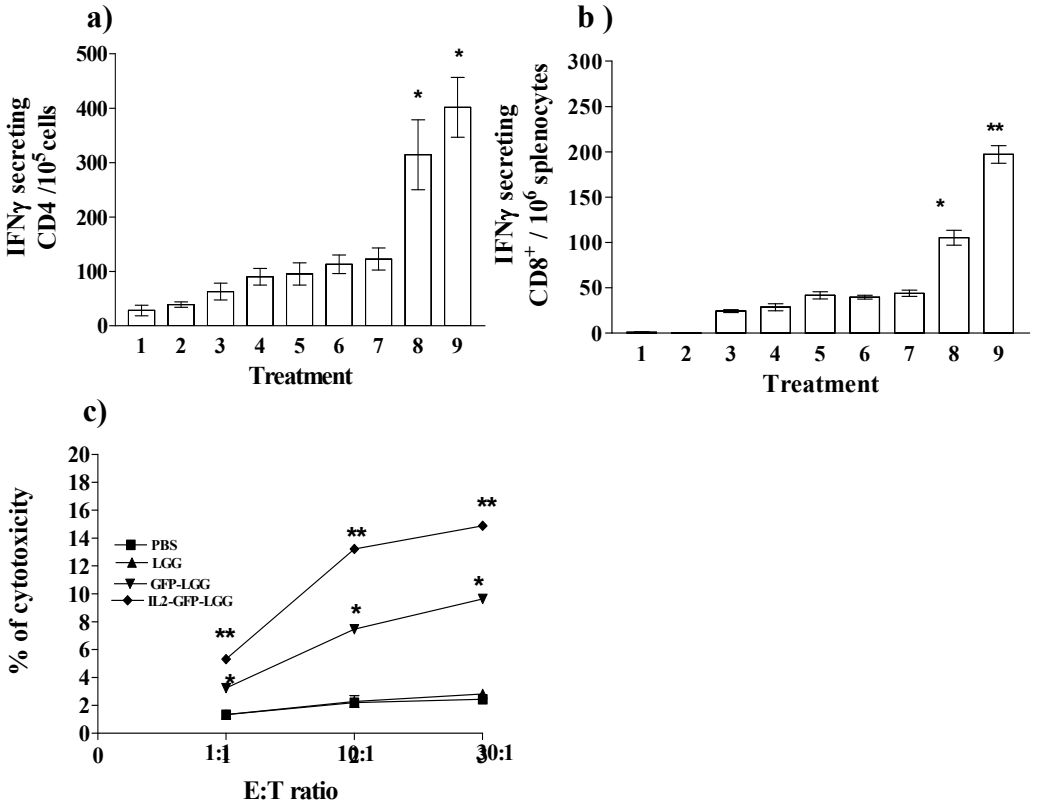


Figure 3.22. IFN-γ ELISPOT for the analysis of GFP specific $CD4^+$ or $CD8^+$ T cells and CTL assay

a) shows GFP specific $CD4^+$T cell responses on day 80. PBS immunized mice splenocytes treated with mitomycin–C cultured alone (column 1); cultured with $CD4^+$ T cells enriched from- PBS immunized mice without (column 2) or with GFP (column 6); LGG immunized mice without (column 3) or with GFP (column 7); LGG-GFP immunized mice without (column 4) or with GFP (column 8) and LGG-IL2-GFP immunized mice without (column 5) or with GFP (column 9). B) shows GFP specific $CD8^+$T cell responses on day 35. The legend is as for $CD4^+$ T cell responses above except that whole splenocytes harvested from immunized mice were co-cultured with mitomycin-C treated splenocytes and GFP specific CTL peptide .

C)) GFP specific CTL responses of splenocytes from mice immunized with modified or wild type LGG. Splenocytes (effector cells) from immunized mice were pooled 7 days after the last booster administration (day 35) and co-cultured with mitomycin C treated MB49-GFP cells and the GFP peptide for 5 days. The MB49-GFP cells (target cells) were mixed with effector cells at various E:T ratios (1:1, 10:1,and 30:1). Specific lysis was determined by quantitative measurements of LDH. * indicates statistical significance from PBS and LGG immunized mice and ** indicates statistical significance from PBS, LGG and LGG-GFP ($p < 0.05$). Data are represented as the mean ± SEM in each group.

Summary III

Intranasal immunization with both modified LGG induced GFP specific serum IgG, IgA, IgM and SIgA. The fusion of IL2 to GFP resulted in significantly increased GFP specific serum IgA and IgG and SIgA titers compared to LGG-GFP immunization. *Lactobacillus* delivery of GFP (approximately 0.1 ng) resulted in approximately 2.0 μg/ml of GFP specific IgG antibodies being produced from LGG-GFP, while co-delivery of IL2 and GFP resulted in more antibody production namely 3.2 μg/ml of GFP specific antibodies. Our data indicates that a low level of secreted IL2 (approximately 20 pg) is sufficient to produce significant adjuvant effects (a 1.6 fold increase in antibody production). Histopathological examination of lung tissues harvested on 35^{th} and 80^{th} day showed no abnormal lung damage happened in nasal immunization of live LGG though increased cellular infiltration was observed on 35^{th} day which was reduced on 80^{th} day. Histopathological examination may be required to be performed 100- 180 days post primary immunization to rule out the persistence of cellular infiltration and chronic lung inflammation. Immunohistochemical staining of the lung tissue showed IgA producing B cells at 80^{th} day of post primary immunization. Nasal delivery of LGG-IL2-GFP induced the expression of chemotactic factors Interleukin-16 (chemotactic factor for $CD4^+$ T cells), chemokine (C-C motif) ligand 25 (CCL25 - chemotactic factor for thymocytes, macrophages and DC) and Interferon γ inducible protein (IP10-chemoattractant for moncytes/macrophages, T cells, NK cells and DC) in lung. Moreover, the expression of chemokine (C-C motif) receptor 2 (CCR2) which is a receptor for monocyte chemoattractant protein-1, a chemokine which

specifically mediates monocyte chemotaxis and CD8a were also upregulated in LGG-IL2-GFP imunization. Increased CD8a mRNA expression indicates increased recruitment of CD8+ T cells to lungs. There were increased GFP specific CD8 T cells in the recall assay which was significantly increased by IL2- GFP nasal delivery.

3.5. *Lactobacilli* secreting IL15/IL2/IL7 and antigen stimulate bone marrow derived dendritic cells and increase antigen specific cytotoxic T lymphocytes responses

The immunomodulatory potential of *lactobacilli* and its ability as a vaccine delivery vehicle are well accepted (Poo *et al.* 2006; Zegers *et al.* 1999) in infection models. In a few cancer models, where the cancer expresses a viral protein or is induced by bacterial infection *Lactobacilli* vaccines have been shown to be efficacious (Corthesy *et al.* 2005; Ho *et al.* 2005; Poo *et al.* 2006). One main problem in cancer is the identification of specific antigens. One of the best studied examples of a cancer marker is the Prostate specific antigen (PSA) which is elevated in prostate cancer.

Cytokines can potentiate the immune response elicited by vaccines but if they promote cytotoxic T lymphocyte (CTL) production as do members of γc cytokine family (IL7, IL15, IL21 and IL2), they may be beneficial for tumour immunotherapy (Fry *et al.* 2002; Ma *et al.* 2003; Mueller *et al.* 2008; Pulendran *et al.* 2004). IL-2 has demonstrated activity against renal cell, melanoma, lymphoma, and leukemia (Niethammer *et al.* 2001; Foa *et al.* 1994). IL15 is pivotal in the development and maintenance of antigen specific $CD8^+$ T cells and co-injection of IL15 gene with a viral DNA vaccine increased CTL responses (Kim *et al.* 1998).

Co-administration of IL15 is known to induce stronger cellular and humoral immune responses systemically and mucosally (Stevceva *et al.* 2006). IL7 has a potential role in modulating peripheral T cell expansion and proliferation in response to high and low affinity antigens (Fry *et al.* 2002). IL21 is more recently identified and in a number of murine tumor models genetically modified to secrete IL21 there was tumor regression or rejection but this response has been linked to NK cell activation rather than T cells (Di Carlo *et al*. 2004; Ma *et al.* 2003). Therefore this study was limited to IL2, IL15 and IL7 to determine their vaccine adjuvant effect using LGG as the delivery vehicle and PSA as the tumor marker.

3.5.1. Increased antigen production with the pLP500slpA promoter plasmid

LGG transformed with the pLP500 plasmid with the *ldh* promoter of *L. casei* upstream of a IL-2-GFP gene fusion produces approximately 1ng of antigen/1.3 x 10^9 CFU/ml in total and about $1/5^{th}$ of this is secreted. To improve antigen production the *ldh* promoter (523bp) was replaced with the slpA promoter from *L. acidophilus* S-layer protein (McCracken *et al.* 2000; Savijoki *et al.* 1997) and its secretory sequence (397 bp). Fig 3.23. shows the slpA promoter region and signal sequence that were amplified and cloned. The cytokine antigen constructs

were produced using this new plasmid as described in Table 2.1.

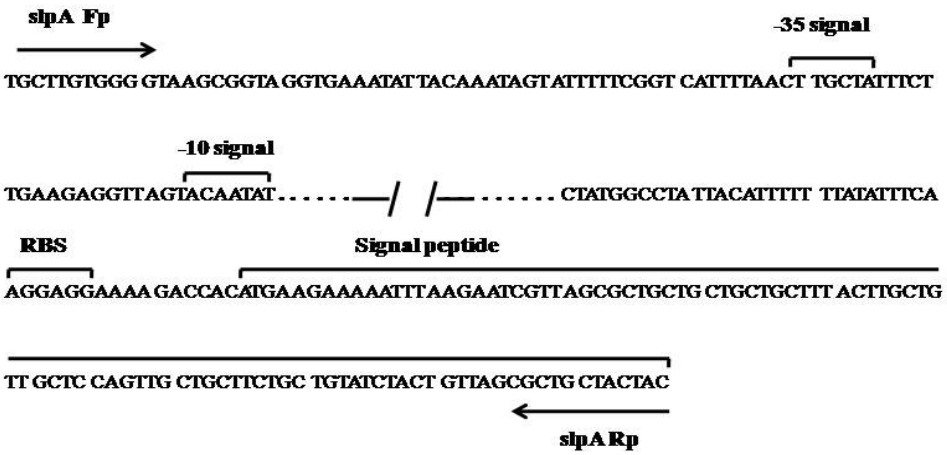


Figure 3.23. Nucleotide sequence of the S-layer protein A promoter region of *Lactobacillus acidophilus* that was amplified and cloned into pLP500. Oligonucleotides slpA FP and slpA RP were used to amplify the promoter region. The location of the -35 and -10 signal, ribosome binding site and signal peptide sequence are indicated on the sequence.

After transformation, LGG Ery+ positive colonies were selected and their growth curves established. Culture supernatants from LGG-S-PSA, LGG-S-IL2-PSA, LGG-S-IL15-PSA and LGG-S-IL7-PSA were harvested at the late exponential phase (approximately after 20-22 hours of culture) and analyzed for PSA or cytokine secretion. The antigen was 100% secreted and the PSA or cytokine-PSA fusion proteins were in the range of 16 - 19 ng/2 x 10^9 CFU/ml which was approximately 10 fold higher than that produced with *ldh* promoter. The biological activity of IL2-PSA and IL15-PSA fusion proteins were measured using a proliferation assay with the IL2 dependent mouse cell line CTLL-2. The biological activity of IL2 or IL15 fused to PSA was not different from IL2 or IL15 secreted without PSA and identical to the activity of the

human IL2 standard.

3.5.2. Both recombinant LGG and control LGG efficiently mature DCs

Previous studies have shown that *Lactobacilli* can induce the up regulation of co-stimulatory molecules in human and mice DCs (Christensen *et al.* 2002; Veckman *et al.* 2004). To determine if this response is modulated by the cytokines, the BMDC were co-cultured with 100 fold more LGG secreting cytokines and antigen and analyzed by flow cytometry (Figure 3.24). All LGG treatment up regulated the expression of CD86, CD80, CD83, CD40 and MHC class II (Table 3.9). LGG-S-IL15-PSA induced statistically significant up regulation of CD80, CD86 and CD40 compared to DC only and DC treated with LGG-S, LGG-S-IL2-PSA and LGG–S-PSA (Table 3.9).

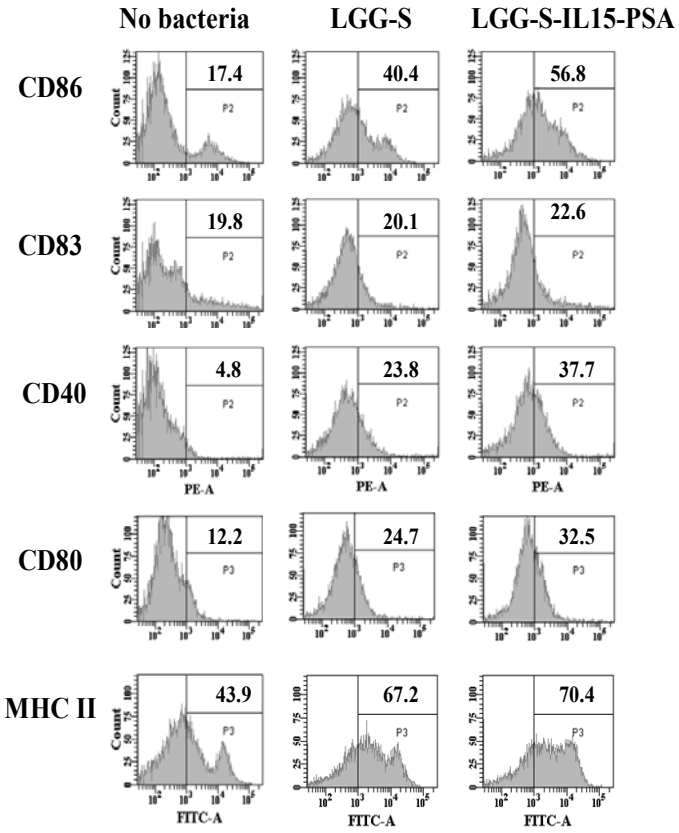


Figure 3.24. ***Lactobacilli*** **induce the maturation of bone marrow derived dendritic cells (BMDC).** BMDC were incubated with LGG-S or LGG-S-IL15-PSA at a bacteria to BMDC ratio of 100:1 overnight and stained with antibodies to co-stimulatory molecules. Flow cytometry histograms are shown for untreated BMDC or BMDC treated with LGG-S or LGG-S-IL15-PSA. The numbers indicate the percentage of cells adjusted by subtraction of the fluorescence by control matched isotype antibodies. The flow cytometry histograms are representative of two independent experiments.

Table 3.9. Expression of maturation markers on dendritic cells after recombinant LGG treatment.

Treatment	% surface expression of co stimulatory receptors				
	CD86	CD80	CD83	CD40	MHC II
BMDC only	16.1 ± 1.29	16.5 ±1.98	24.7 ± 2.09	7.0 ± 0.94	53.8 ± 1.71
DC+LGG (SlpA)	52.5 ± 2.93	32.4 ± 2.86	38.7 ± 3.28	41.6 ± 2.92	67.4 ± 2.34
DC+ LGG (IL2-PSA)	60.4 ± 6.03	29.1 ± 3.54	38.7 ± 3.11	45.4 ± 3.81	67.3 ± 0.9
DC+LGG (IL15-PSA)	61.4 ± 6.17	39.3 ± 3.22*	38.0 ± 1.76	52.1 ± 2.00**	68.2 ± 1.77
DC+LGG (IL7-PSA)	58.1 ± 2.59	38.3 ± 3.61*	38.5 ± 2.42	46.5 ± 2.03	68.4 ± 1.20
DC+LGG (PSA)	61.8 ± 3.39	35.8 ± 2.26	37.5 ± 1.81	47.8 ± 2.02	68.6 ± 1.37

* Statistically significant ($p<0.05$) compared to DC only and DC+LGG (IL2-PSA).
** Statistically significant compared to DC only, DC+ LGG(SlpA) and DC+LGG(IL2-PSA).

* Statistically significant ($p < 0.05$) compared to DC only and DC + LGG (IL2-PSA). ** Statistically significant compared to DC only, DC + LGG(SlpA) and DC + LGG(IL2-PSA).

3.5.3. LGG–S-IL15-PSA induces more IL12p70 production by BMDCs

DCs are known to secrete IL12 when cultured with *Lactobacillus* species (Mohamadzadeh *et al.* 2005). We analyzed the effect of the cytokines on the induction of IL12p70 (bioactive IL12) by BMDC. Figure 3.25.a, shows the level of IL12p70 produced during BMDC-bacteria co-culture. All the cytokine-PSA secreting LGG induced statistically significant IL12 production compared to control LGG (LGG-S) or LGG-S-PSA. IL15-PSA secreting LGG induced a statistically significant increase in IL12 (about 670 pg/ml) production compared with IL2 or IL7-PSA secreting LGG. However, there was no difference in the levels of TNFα produced after stimulation of BMDCs by any of the modified LGG as seen in Figure 3.25.b. IL15 and IL2-PSA secreting LGG suppressed IL10 secretion by BMDC and it was statistically significant with IL10 induced by control LGG or IL7-PSA or PSA secreting LGG (figure 3.25.c).

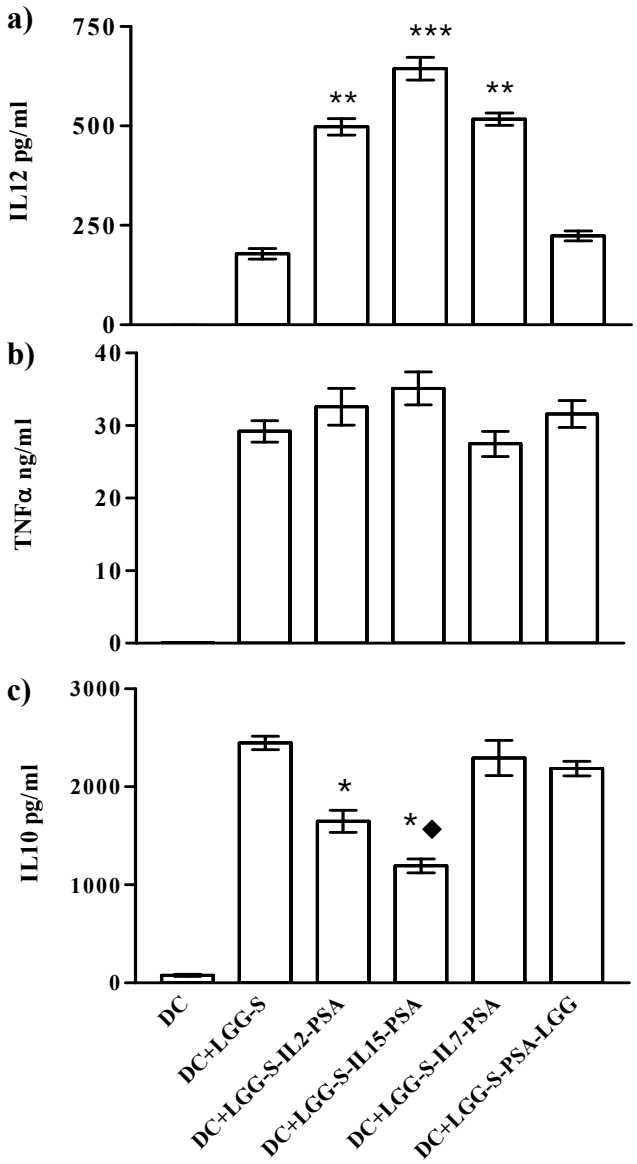


Figure 3.25. Induction of IL12p70, TNFα and IL10 production by BMDCs treated with recombinant LGG. BMDCs were treated with recombinant LGG overnight and analyzed for IL10, IL12p70 and TNFα by ELISA. * denotes statistical significance from DC, DC+ LGG-S and DC+ LGG-S-IL7-PSA. *♦ denotes statistical significance from DC, DC+ LGG-S, DC+ LGG-S-IL7-PSA and LGG-S-PSA. ** denotes statistical significance from DC, DC+ LGG-S and LGG-S-PSA. *** denotes statistical significance from DC, DC+ LGG-S, DC + LGG-S-IL2-PSA, DC+LGG-S-IL7-PSA and LGG-S-PSA. A $p < 0.05$ is statistically significant. Data are represented as mean ± SEM for triplicates of two independent experiments.

3.5.4. Induction of T cell proliferation and activation by BMDC mediated antigen presentation

DC induce the adaptive immune response by priming T cells to proliferate and secrete IL2 or IFNγ. Priming of T cells depends on antigen presentation by MHC I or II complexes on antigen presenting cells. BMDC were incubated overnight with either cytokine–PSA secreting or PSA secreting or control LGG. External bacteria were killed and cleared and the BMDC were incubated with CFSE labeled or unlabeled naïve T cells for 5 days for antigen priming. T cell proliferation was observed in co-culture with DCs treated with LGG-S or LGG-S-IL15-PSA compared to untreated DC, Figure 3.26. a - d.

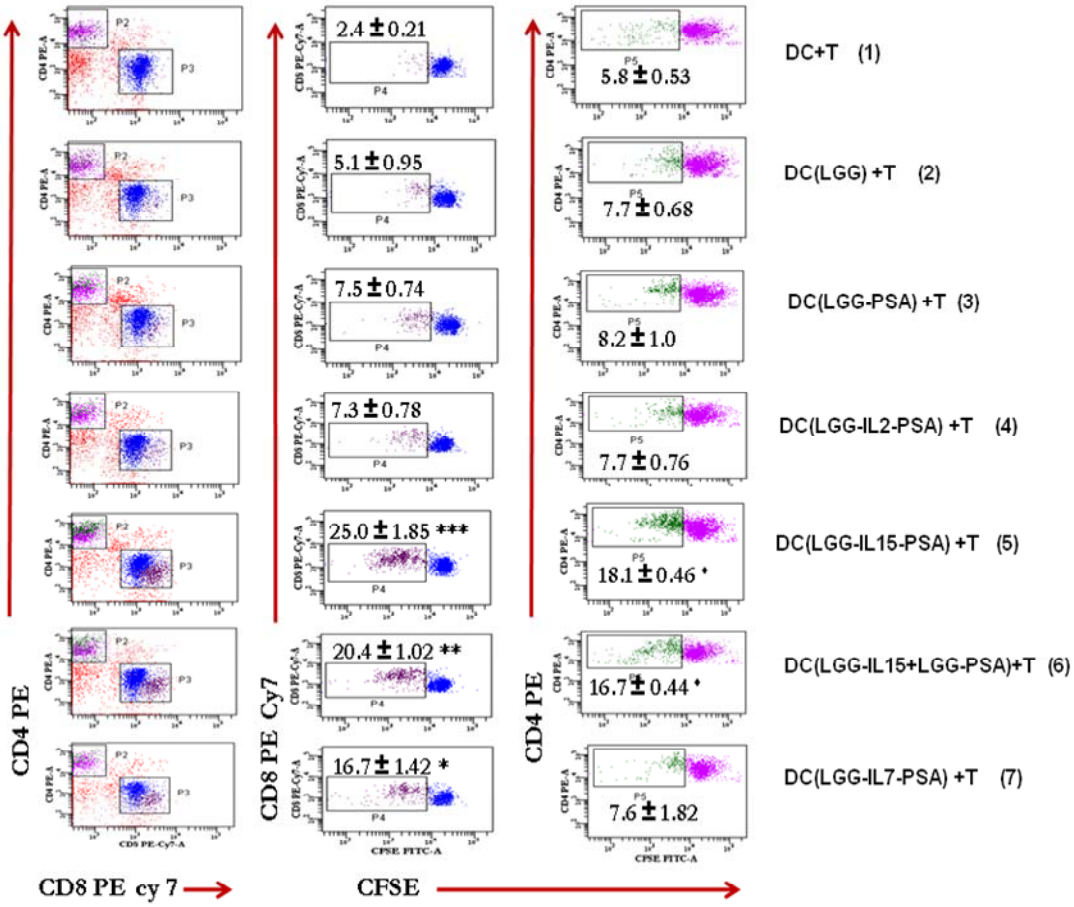


Figure 3.26. Induction of T cell proliferation by DC stimulated with lactobacilli. BMDC were treated with LGG-S or LGG-S-IL15-PSA overnight at a bacteria to BMDC ratio of 100:1 and then the bacteria were killed by gentamycin treatment. The DCs were then co-cultured with CFSE labelled naive T cells at a stimulator to responder ratio of 1:100 for 3 days. Proliferation of $CD4^+$ or $CD8^+$ T cells were analyzed by flowcytometer. For $CD8^+$ T cells proliferation *** denotes statistically significant with 1,2,3,4,6&7,** statistically significant with 1,2,3,4&7 and statistically significant with 1,2,3 &4. For $CD4^+$ T cells proliferation ♦ denotes statistically significant with 1,2,3,4 &7.

IL15-PSA secreting LGG induced a high allogeneic $CD8^+$ T cell proliferation which was statistically significant with LGG or PSA secreting LGG or IL2/IL7-PSA secreting LGG treated groups. Though the $CD8^+$ T cells co-cultured with LGG-PSA and LGG-IL15 treated DC also demonstrated a high proliferation rate compared to other treatment groups, it was not as high as the proliferation observed with LGG-IL15-PSA treatment which highlighted the enhanced antigen presentation obtained with fusion construct (Figure 3.26).

LGG itself stimulated DC to activate T cell IL-2 production and this was increased in the presence of antigen and was further enhanced by the presence of IL15. LGG-S-IL15-PSA treated DC induced T cells to secrete a higher level of IL2 ($p < 0.05$) than LGG-S (Figure 3.27.a). All PSA/cytokine-PSA secreting LGG treated DC cultured with T cells elicited significant IFNγ production ($p < 0.05$) compared to LGG-S. IFNγ production induced by LGG-S-IL15-PSA was significantly more than that produced after stimulation with LGG-S-IL2-PSA or LGG-S-PSA (figure 3.27.b).

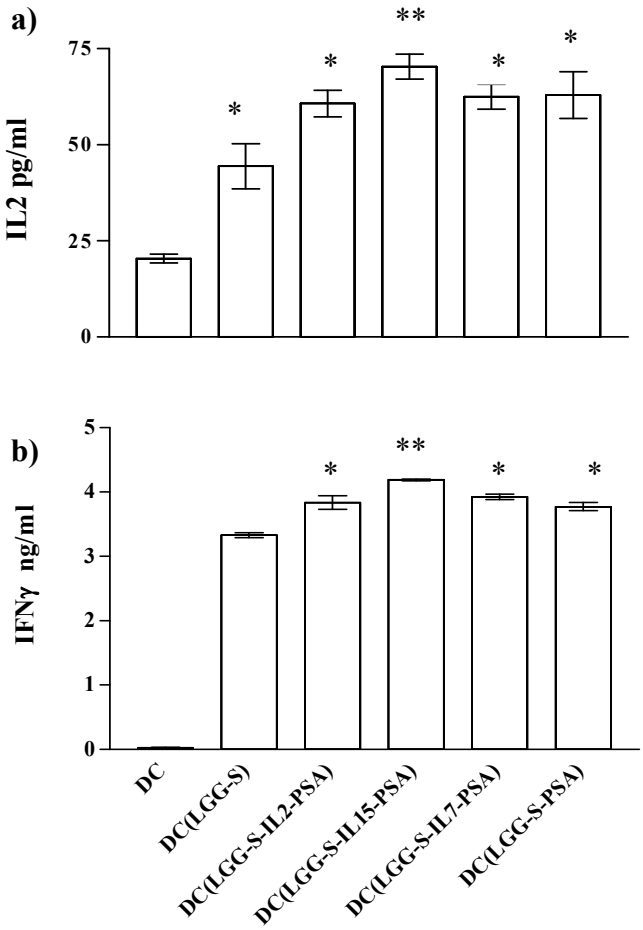
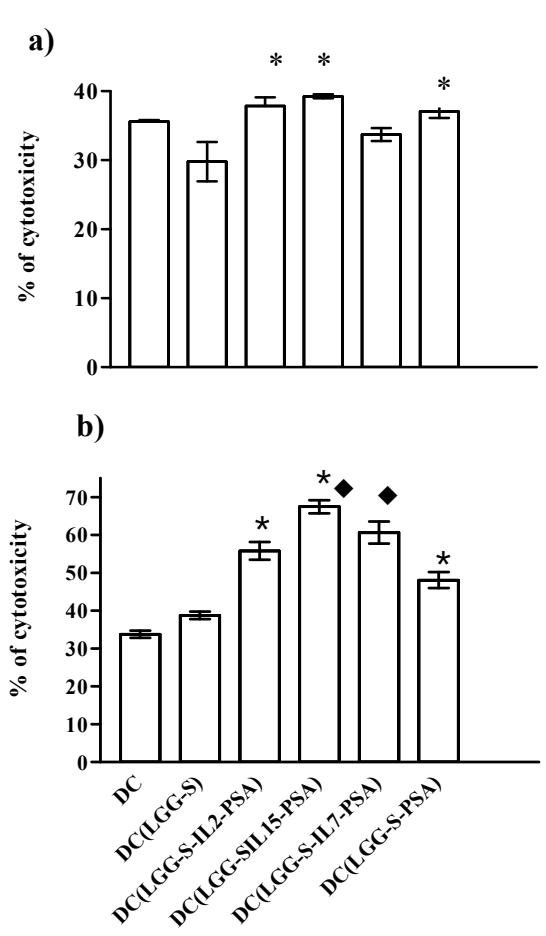


Figure 3.27. **LGG itself stimulated DC to activate T cell IL-2 production and this was increased in the presence of antigen and was further enhanced by the presence of IL15**. LGG-S-IL15-PSA treated DC induced T cells to secrete a higher level of IL2 ($p < 0.05$) than LGG-S (a). All PSA/cytokine-PSA secreting LGG treated DC cultured with T cells elicited significant IFNγ production ($p < 0.05$) compared to LGG-S. IFNγ production induced by LGG-S-IL15-PSA was significantly more than that produced after stimulation with LGG-S-IL2-PSA or LGG-S-PSA (b).

* denotes statistical significance ($p < 0.05$) from untreated DC-T co-culture and
** denotes statistical significance from untreated DC-T and LGG -S treated DC-T cells co-culture. Data are represented as mean ± SEM of experiments

3.5.5. Antigen specific cytotoxicity assay

To determine the functional activity of T cells the T cells were harvested after 5 days of co-culture and incubated with the target tumor cells MB49-PSA or control MB49 cells. The LDH activity was measured in the supernatant after 5 hours incubation. DC primed T cells (with or without bacterial stimulation) showed a non-specific cytotoxic effect on control MB49 (about 30 - 40%), figure 3.28 (a) T cells primed with DC that had been stimulated with PSA or cytokine–PSA secreting bacteria demonstrated a higher cytotoxic effect on MB49-PSA cells (about 50 - 70%), figure 3.28 (b). LGG-S-IL15-PSA treated DC primed T cells to kill significantly more MB49-PSA cells than LGG-S-IL2-PSA ($p < 0.05$) as seen in figure.3.28 (b). To determine if the fusion protein of IL15and PSA was as effective as IL15 and PSA secreted singly, LGG expressing each gene singly were also produce. Interestingly, LGG-S-IL15-PSA (fusion protein) treated DC primed T cells showed a higher cytotoxic effect on MB-49-PSA or MB-49 than LGG-S-IL15 + LGG-S-PSA treated DC primed T cells as seen in figure 3.28.(c).



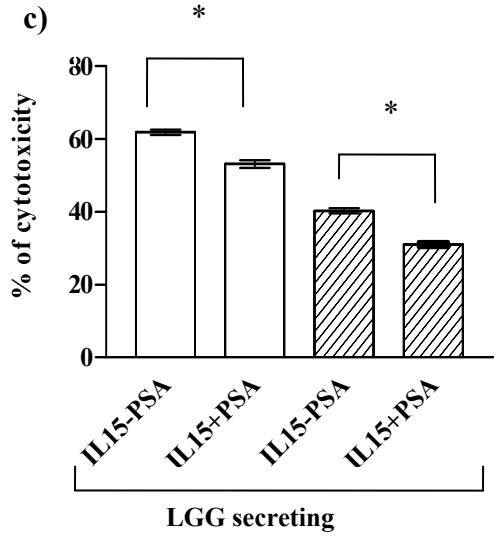


Figure 3.28. Recombinant LGG stimulated DC efficiently prime naïve T cells and generate antigen specific CD8+ T cells.

a) and b) T cell mediated cytotoxicity on MB49 or PSA secreting MB49 target cells respectively. Target cells were added to 5 days DC-T cell co-cultures and LDH activity was measured in the supernatant after 5 hours of incubation. % of cytotoxicity was calculated from LDH activity. c) LGG-S-IL15-PSA or LGG-S-IL15+LGG-S-PSA treated DC primed T cell mediated cytotoxicity on target cells MB49PSA. DC stimulated overnight with LGG-S-IL15-PSA or LGG-S-IL15+LGG-S-PSA were co-cultured with naïve T cells for 5 days and target cells MB49 PSA (clear) or MB 49 (cross striped) were added. LDH activity was measured in the supernatant after 5 hours

*Denotes statistical significance ($p<0.05$) from untreated DC-T and LGG-S treated DC-T cells co-culture, *♦ denotes statistical significance from DC cultured with T cells or DC cultured with T cells after stimulation with LGG-S, LGG-S-IL2-PSA and LGG-S-PSA. ♦ denotes statistical significance from DC cultured with T cells or DC cultured with T cells after stimulation with LGG-S and LGG-S-PSA. Data are represented as mean ± SEM of experiments performed independently twice in triplicates.

Summary IV

Improved antigen production was seen in LGG using the slpA promoter and secretory signal and that cytokines especially IL15 enhanced antigen presentation and activation of T cells. The cytotoxic T cells produced by DCs primed with LGG secreting PSA or cytokine PSA fusion proteins specifically recognized tumour cells secreting PSA.

All the antigens produced were secreted unlike LGG transformed with the PLP500 plasmid with the ldh promoter, where most of the antigen produced remained within the bacteria. IL15-PSA or IL2–PSA secreting LGG reduced IL10 production by DC, IL7 did not, but all three resulted in increased IL12p70 production. However, the T cell response did not correlate with differences in IL12 or IL10 production.

LGG-S-IL15-PSA treated DC showed high IFNγ production and CTL response on target cells indicating efficient antigen presentation to T cells. The increased antigen specific cytotoxicity rendered by the T cells primed with DC that had been stimulated with IL15-PSA secreting LGG was statistically significant from combined stimulation with IL15 or PSA secreting LGG. Thus our data indicate that LGG as a promising antigen delivery vehicle and that IL15 is a good vaccine adjuvant especially when administered as fusion protein with antigen. It also indicates that formation of the fusion protein induces a better response.

3.6. Cross talk between LGG treated neutrophils and dendritic cells and its effect on DC activation and antigen presentation

After intranasal immunization we observed neutrophils recruitment to the lungs. Neutrophils are effective phagocytic scavengers and the first line of defense against invading microbes. During infections, polymorphonuclear leukocytes are the first cell type to arrive to the infection site and upon encountering bacteria, neutrophils release the generation of reactive oxygen intermediates, preformed lytic enzymes and inflammatory mediators to kill the bacteria. Infected neutrophils are programmed to die by apoptosis and subsequently are removed by macrophages or dendritic cells which are also chemoattracted to the infection site. At the site of inflammation activated neutrophils and DCs are shown to interact positively through Mac-1/DC-SIGN molecules expressed by neutrophils and dendritic cells respectively.

Even after the clearance of the bacteria at the infection site, live or apoptotic neutrophils could transfer the antigen to DC allowing the latter to stimulate the T cells to secrete IL2 or IFNγ. In intranasal immunization, neutrophils would internalize the recombinant LGG and transfer the antigen to DC to render the antigen specific immune response. In this study, we analyzed the cross talk between LGG treated neutrophils and dendritic cells and its effect on DC activation and antigen presentation to T cells.

3.6.1. IL10 and TNFα predominantly produced in LGG stimulated neutrophils culture.

Bone marrow derived neutrophils were treated with IL15-PSA or PSA secreting LGG or LGG-S for 18hours. Culture supernatants were analyzed for

IL12p70 or IL10 or TNFα. Lactobacilli stimulated neutrophils produced bioactive IL12, IL10 and TNFα (Table 3.10).

Table 3.10. Cytokines produced from neutrophils on LGG treatment for 18 hours

Treatment	level of cytokines (pg/ml)		
	IL10	IL12 p70	TNFα
Neutrophils only	0	19.4 ± 13.66	14.4 ± 4.57
Neutrophils + LGG-S	1632.8 ± 129.38*	117.3 ± 5.37	1314.0 ± 66.49
Neutrophils + LGG-S-IL15-PSA	1601.4 ± 133.48	131.0 ± 20.36	1357.0 ± 99.27
Neutrophils + LGG-S-PSA	1401.4 ± 61.32	123.5 ± 24.56	1391.0 ± 89.85

Data represent mean ± standard deviations

* Statistically significant ($p < 0.05$) compared to neutrophils treated with LGG-S-PSA or neutrophils only.

3.6.2. Induction of T cell activation by bone marrow derived neutrophil (BMN) mediated antigen presentation

Neutrophils induce the adaptive immune response by activating T cells to secrete IL2 or IFNγ. Unlike the DC, neutrophils stimulated with recombinant *lactobacilli* did not show a higher level of MHC II, rather they showed a higher level of MHC I expression. MHC I expression on unstimulated BMN was about 37% whereas all recombinant LGG stimulated BMN showed the expression in the range of 64 - 67%. BMN were incubated for 18 hours with either IL15–PSA secreting or PSA secreting LGG or control LGG. External bacteria were killed and cleared and the BMDC were incubated with allogeneic T cells for 2 days for activation. IL2 produced from LGG-S-IL15-PSA or LGG-S-PSA treated

neutrophils induced T cells was not significantly different from LGG-S stimulated neutrophils – T cell co-culture as seen figure 3.29.a. However LGG-S-PSA or LGG-S-IL15-PSA treated neutrophils induced a higher level of IFNγ ($p < 0.05$) than LGG-S. IFNγ production induced by LGG-S-IL15-PSA was significantly more than that produced after stimulation with LGG-S-PSA ($p < 0.05$) as seen in figure 3.29.b.

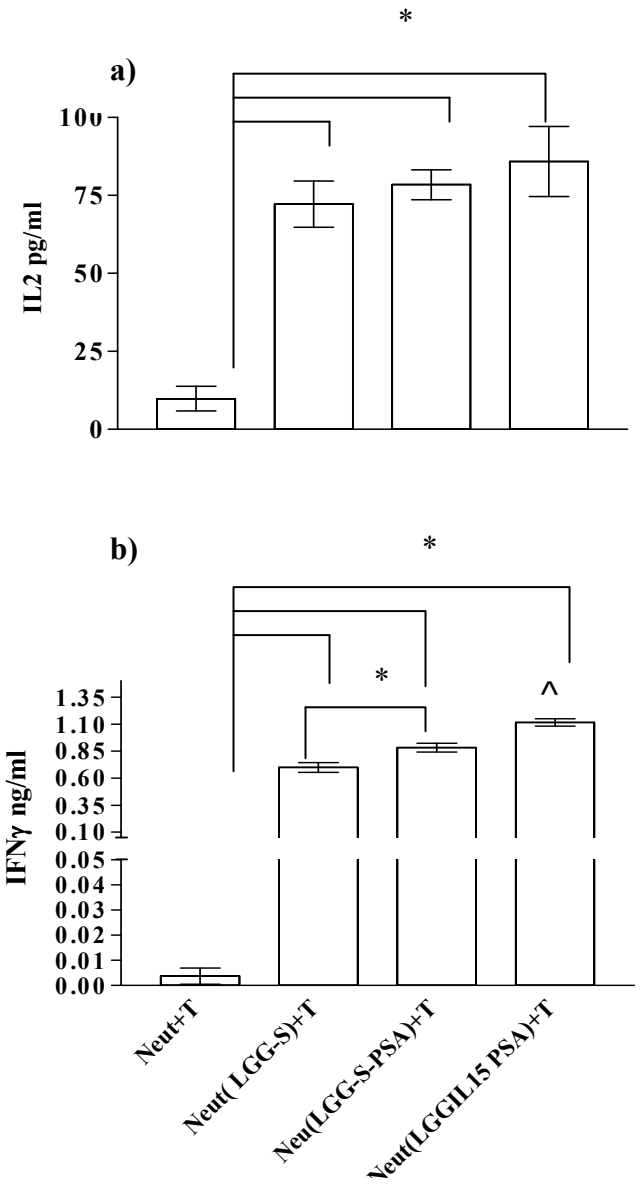


Figure 3.29. LGG treated neutrophils induce T cells proliferation

Unstimulated neutrophils or overnight stimulated neutrophils with LGG-S or LGG-S-IL15-PSA or LGG-S-PSA were co-cultured with allogeneic T cells for 2 days. After 2 days culture supernatants were harvested and analyzed for IL2 (a) or IFNγ (b). *denotes statistically significant and ^ denotes statistical significance from unstimulated neutrophils or LGG-S treated neutrophils – T cells co-culture or LGG-S-PSA treated neutrophils-T cells co-culture. ($p<0.05$). Data are represented by the mean ± SEM in each group. Data are representative of three different experiments.

3.6.3. Impact of LGG stimulated neutrophils on DC activation

We investigated neutrophils-DC crosstalk in response to LGG. For the neutrophils treatment LGG-S and LGG-S-IL15-PSA were used. Neutrophils were stimulated with LGG-S or LGG-S-IL15-PSA for 2 hour. After extracellular bacteria were killed by gentamycin treatment and subsequent PBS wash, cells were incubated with BMDC for 16 hours. For control, neutrophils or BMDC were stimulated with rLGG for 2 hours and incubated for 18 hours in fresh media after gentamycin treatment and PBS wash. We analyzed whether LGG stimulated neutrophils could activate immature DC.

Co-stimulatory molecules: CD86, CD80, CD83, CD40 and MHC II were significantly up regulated on DC in all LGG stimulated neutrophils-DC co-culture as seen in Table 3.11.

Table 3.11. LGG treated neutrophils upregulate co-stimulatory molecules on DC

Treatment	% of co stimulatory molecules expression on DC				
	CD86	CD80	CD83	CD40	MHC II
Neut+DC only	3.69 ± 3.2	5.57 ± 2.1	11.64±2.8	6.29±3.4	40.3± 3.2
DC[LGG-S]	65.4 ± 1.2*	29.4± 3.1*	28.23±2.8*	57.4±1.9*	68.2 ± 3.2*
DC+Neut [LGG-S]	73.9 ± 3.2*	33.3±2.1*	28.57±3.1*	55.8±2.4*	75.25±2.2*
DC+ Neut [LGG-PSA]	72.55 ± 0.9*	35.6±1.2*	25.81±0.7*	61.21±4.1*	73.33±3.2*
DC+ Neut [LGG-IL15-PSA]	76.91 ± 3.1*	35.7±2.9*	28.99±3.1*	61.51±4.2*	75.76±2.0*

Data represent mean % of cells ± standard deviations
* Statistically significant ($p < 0.05$) compared to unstimulated Neutrophils - DC co culture.

3.6.4. LGG treated neutrophils differentially affect cytokine production by DC Direct interaction of LGG with BMDC or neutrophils produced higher IL12p70 or IL10 respectively. LGG-S-IL15-PSA stimulation induced significantly higher level of IL12 than that produced by LGG-S. However LGG stimulated neutrophils downregulate IL12p70 and increase IL10 production from BMDC in BMDC-neutrophils co-culture. This up regulated IL10 or downregulated IL12 production from BMDC induced by LGG stimulated neutrophils was significantly different from IL10 or IL12 produced from neutrophils or BMDC that were directly stimulated with LGG (figure 3.30. a&b).

TNFα was highly produced from DC or neutrophils upon LGG stimulation. LGG-S or LGG-S-IL15-PSA induced almost the same level of TNFα from DC in direct stimulation. But in indirect stimulation through neutrophils, TNFα production was enhanced or suppressed depending on whether LGG-IL15-PSA or LGG-S was used as seen in figure 3.30.c.

Unstimulated DC produced a high level of TGFβ by itself. Upon LGG stimulation, TGFβ production was significantly reduced from DC and interestingly, neutrophils could elevate TGFβ production when co cultured with DC. The level of TGFβ production from DC co-cultured with neutrophils which had been stimulated with LGG-S or LGG-S-IL15-PSA was significantly inhibited compared to DC co cultured with untreated neutrophils. (Figure 3.30.d). Moreover, TGFβ production from LGG-S or LGG-S-IL15-PSA treated neutrophils-DC co-culture was statistically significant from neutrophils or DC individually stimulated with LGG-S or LGG-S-IL15-PSA respectively.

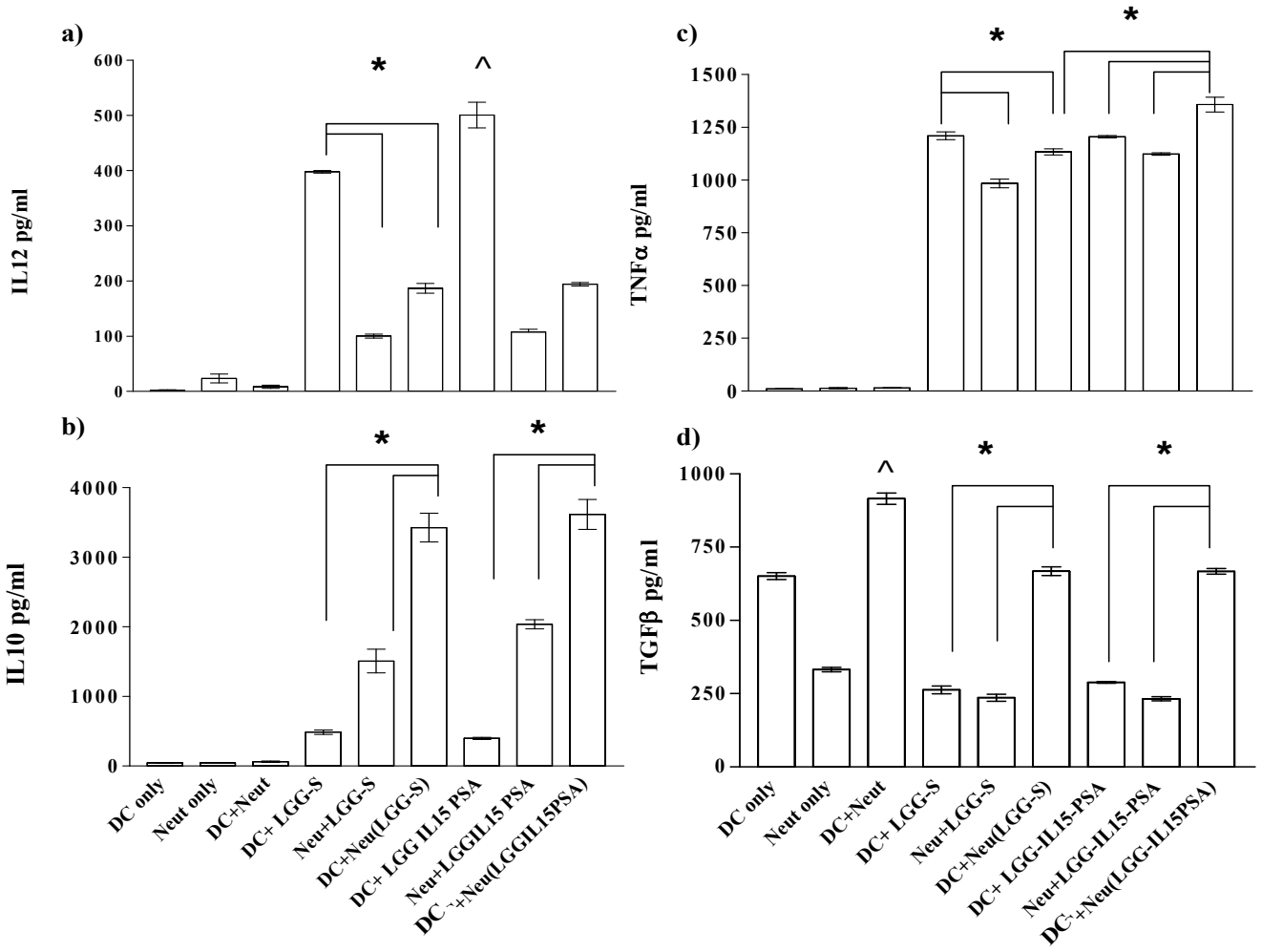


Figure 3.30. Bioactive IL12 or IL10 or TNFα and or TGFβ levels in LGG treated BMN or BMDC or LGG treated BMN-BMDC co culture .

BMN or BMDC were treated with LGG-S or LGG-S-IL15-PSA for 2 hours before the bacteria were killed and the treated BMN co cultured with BMDC overnight. Culture supernatants were harvested and analyzed for IL12p70 (a) or IL10 (b) or TNFα (c) and or TGFβ (d). Data represents three separate experiments. Data are represented by the mean ± SEM in each group.
* denotes statistical ignificance,
^ denotes statistically ignificant with all groups. P value < 0.05.

3.6.5. DC co-cultured with recombinant LGG treated neutrophils elicit T cells to produce anti-inflammatory cytokines

We investigated whether the DC mediated T cell antigen presentation through neutrophils could be different from direct antigen presentation. In direct antigen presentation, DC mediated antigen presentation was more efficient than neutrophils mediated presentation with respect to IL2 or IFNγ production. However, LGG-S not LGG-S-IL15-PSA treated neutrophils mediated indirect antigen presentation in DC induces a significantly higher IL2, which was statistically significant from LGG-S treated DC mediated direct antigen presentation. But IFNγ from T cells in direct antigen presentation was not different from neutrophils mediated indirect antigen presentation as seen in Figure 3.31. a&b.

Direct DC mediated antigen presentation actually suppressed TGFβ production which is in contrast with enhanced TGF β production observed in neutrophils mediated antigen presentation. DC mediated antigen presentation to T cells after culture with neutrophils engenders an anti-inflammatory milieu which was represented by a significantly higher TGFβ and IL10, Figure 3.31 c & d.

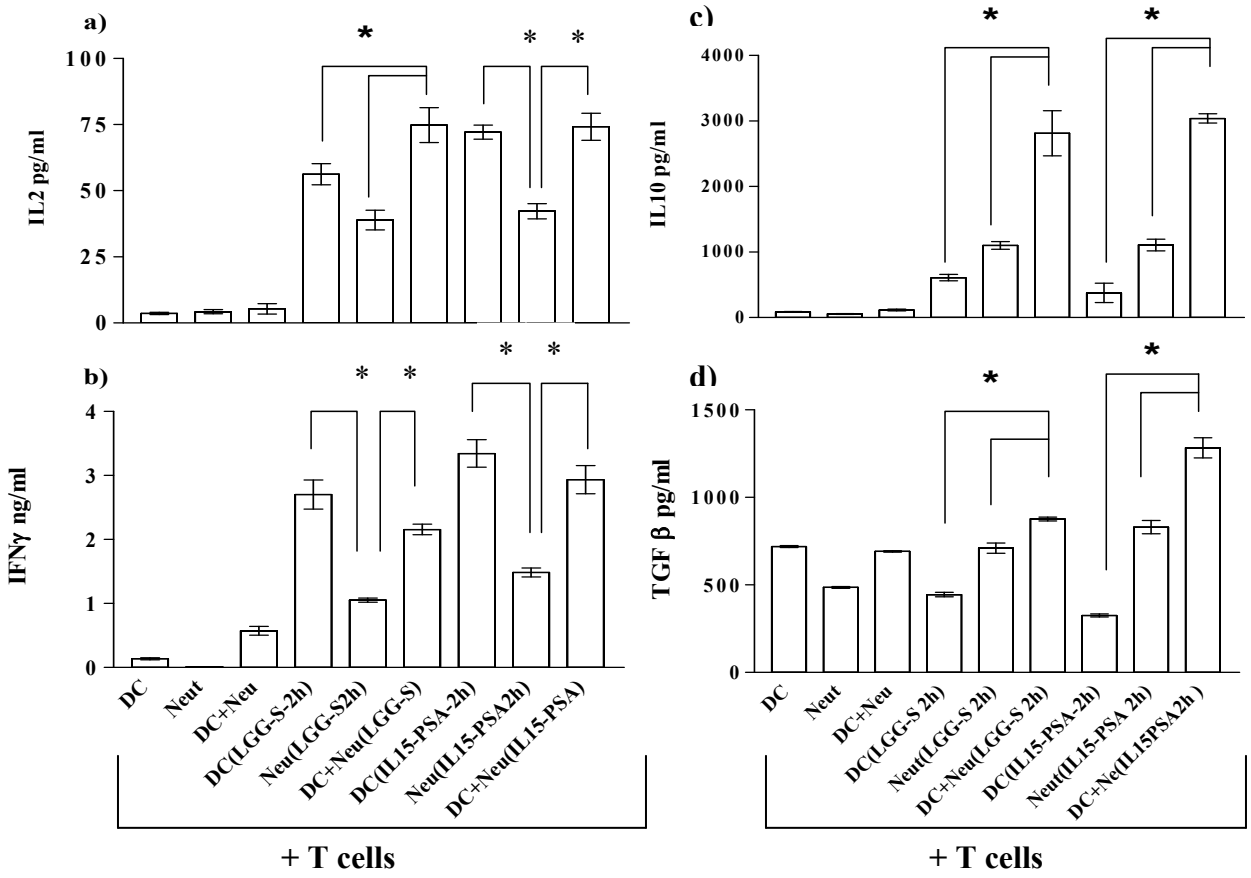


Figure 3.31. Cytokine production by neutrophils mediated direct or indirect antigen presentation through DC to allogeneic T cells.

BMN or BMDC were treated with LGG-S or LGG-S- IL15-PSA for 2 hours and treated BMN were co-cultured with BMDC overnight followed by addition of allogeneic T cells and incubated for 48 hours. Culture supernatants were harvested and analyzed for IL2 (a); IFNγ (b); IL10(c) and TGFβ (d).

Data represents two separate experiments. Data are represented by the mean ± SEM in each group.

*denotes statistically significant ($P < 0.05$).

3.6.6. Study of antigen specific cytotoxic T cells generated by neutrophil indirect antigen presentation through DC

The differential cytokine profile observed in neutrophil antigen presentation through DC may influence antigen specific T cell cytotoxicity. After bacteria treated neutrophils-DC co-culture, DCs were enriched using CD11c microbeads and co-cultured with naïve T cells for 5 days to generate PSA specific T cells. Then target tumour cells MB49-PSA or control MB49 were added to study the cytotoxic effect. PSA specific cytotoxicity was calculated based on the LDH released to the culture supernatant. LGG-IL15-PSA or LGG-PSA treated DC were able to generate a high number of antigen specific T cells as the % of cytotoxicity observed in DC mediated direct antigen presentation is higher (40.9 ± 0.78 or 31.0 ± 1.09) compared to neutrophils mediated indirect DC antigen presentation (23.8 ± 1.77 or 21.7 ± 1.55). Another interesting feature, 2 hours LGG-IL15-PSA treated DC elicited a significant cytotoxic effect compared to LGG-PSA treated DC (Fig 3.32 a&b).

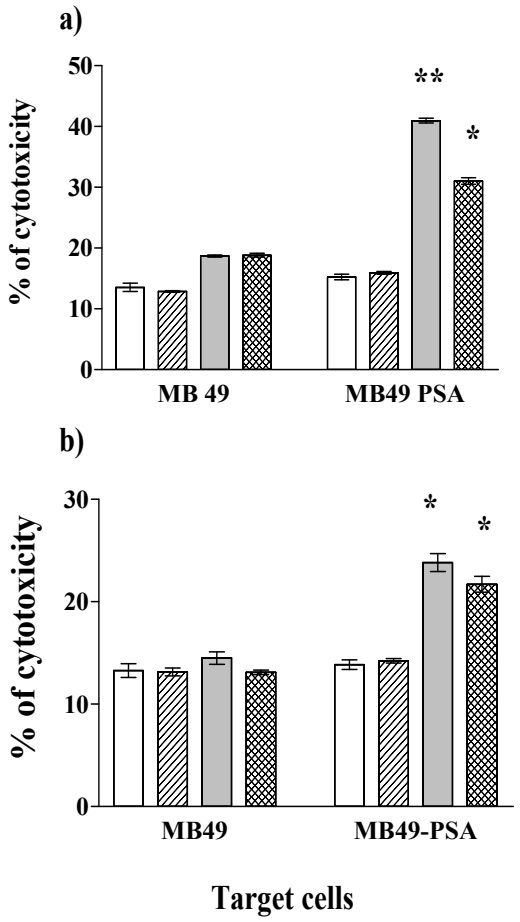


Figure 3.32. Antigen specific cytotoxicity of the T cells generated by co-culture with DC treated with recombinant LGG for 2 hours (a) or DC enriched from overnight culture of DC+ LGG treated neutrophils (b). Neutrophils or DC treated with LGG-S (striped) or LGG-IL15-PSA (shaded) or LGG-PSA (cross striped) or left untreated (clear) and used for DC co-culture followed by T cell presentation assay or T cells co-culture respectively. PSA secreting (MB49-PSA) or control mouse urothelial cancer cell line (MB49) was used as target cells. Data are represented by the mean ± SEM in each group. * Statistically significant ($p < 0.05$) compared to untreated or treatment with LGG-S. ** Statistically significant compared to untreated or treatment with LGG-S or LGG-PSA.

Summary V

LGG treatment with neutrophils did produce bioactive IL12 and a high level of IL10 and TNFα. Moreover, LGG treated neutrophils did not induce any of the co-stimulatory molecules or MHC II expression, but only showed elevated expression of the MHC I molecules. In antigen presentation even though

overnight stimulated LGG efficiently induced allogeneic T cells proliferation, 2 hours stimulated neutrophils elicited 1.05 or 1.49 ng of IFNγ which are slightly higher than 0.70 or 1.11 ng produced in overnight stimulation with LGG-S or LGG-S-IL15-PSA treatment respectively. Increased expression of co-stimulatory molecules on DC in neutrophils-DC co-culture clearly showed bacteria treated neutrophils could deliver the maturation signals to immature DC. However, LGG treated neutrophils stimulate higher IL10 but not IL12 from DC. High IL10 along with increased production of TGFβ was observed in DC-LGG treated neutrophils co-culture. However it did not affect the T cell presentation very much that was characterized by high IL2 and IFNγ which were almost the same level as the DC or much higher than neutrophils mediated direct antigen presentation. But the cytotoxic effect elicited by the T cells generated by co-culturing with recombinant LGG treated DC was higher than that with the DC that were co-cultured with recombinant LGG treated neutrophils.

3.7. Improvement of antigen production in LGG using different promoters

Though high antigen production was not considered as a prerequisite for colonizing lactic acid bacteria in mucosal immunization, high antigen producing bacteria will enhance the antigen specific immune induction. Our initial experiment done with LGG transformed with pLP500-IL2-GFP plasmid which has lactate dehydrogenase promoter (*ldh*) promoter, expressed only about 1-2ng antigen. Later experiments done with the replacement of *ldh* with S-layer protein A (*slpA*) promoter in the plasmid increased the antigen expression to 20ng. However it was not as high as previously reported using *lactobacillus brevis* S-layer signal (Savijoki *et al.* 1997). Recombinant protein production normally depends on the strength of the promoter of the plasmid, plasmid copy number and the target strain. The knowledge of stable or strong promoters in LGG was limited since LGG's complete genome sequence is not known and LGG is infrequently used as a vaccine vehicle to express foreign protein. In this study two strategies were attempted to improve the protein expression as seen in Figure 3.33.

1. To express the protein using a tandem promoter contruct.

An enhanced gene expression under two tandem promoters was reported in *Lactococcus lactis* (Wei *et al*, 2002). We cloned two promoters, *ldh* or *slpA* separately or tandemly by integrating the core *ldh* promoter without ribosome binding site with *slpA* promoter.

2. To use the phosphoglycerate mutase (pgm) promoter of *L. acidophilus*.

Promoter pgm was cloned with its secretory signal peptide sequence in pLP500 plasmid after *ldh* promoter was removed by restriction digestion.

Mouse IL2 cDNA was cloned in either plasmid and the efficiency of protein expression was analyzed.

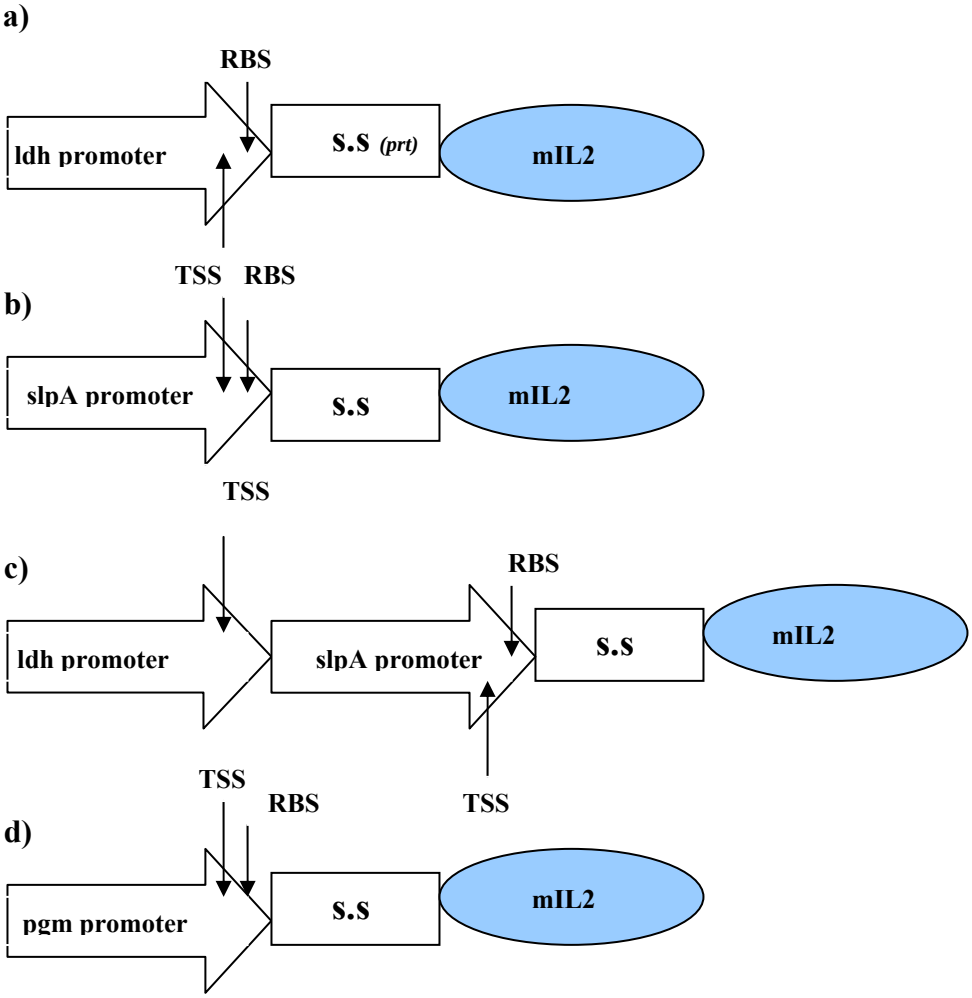


Figure 3.33. Construction of plasmids that secrete murine IL2 under different promoters. a) or b) are IL2 secreting plasmids under ldh or slpA promoters. c) IL2 secreting plasmid under ldh-slpA tandem promoter. d) IL2 secreting plasmid under putative promoter of pgm gene. S.S- secretory sequence of slpA promoter. TSS-transcription start site. RBS- ribosome binding site. S.S (prt)- secretory sequence of proteinase gene.

3.7.1. Construction of pLP500$_{ldh\text{-}slpAp}$ plasmid

The core promoter region without ribosome binding site of *ldh* (figure.3.34) was amplified from *L. caesei* genomic DNA by PCR using the primers listed in Table. 2.2 and cloned in pLP500-slpAp plasmid to give pLP500ldh- slpAp .

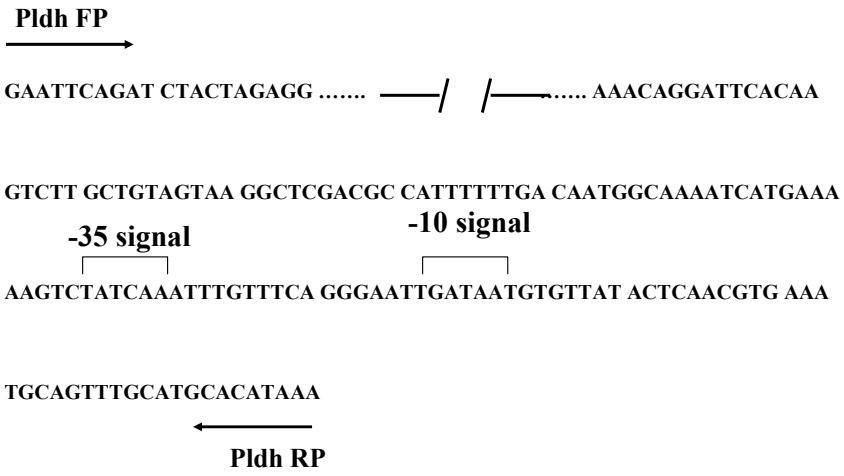


Figure 3.34. Nucleotide sequence of the *Lactobacillus caesei* ldh core promoter without RBS. Oligonucleotides Pldh FP and Pldh RP were used to amplify the promoter region. The location of the -35 and -10 signal are indicated on the sequence.

3.7.2. Construction of pLP500pgmp plasmid

A putative promoter of pgm gene of *L. acidophilus* (Figure 3.35) was amplified from genomic DNA by PCR using the primers listed in Table. 2.2 and cloned in to Pldh removed pLP500 plasmid to generate pLP500pgmp.

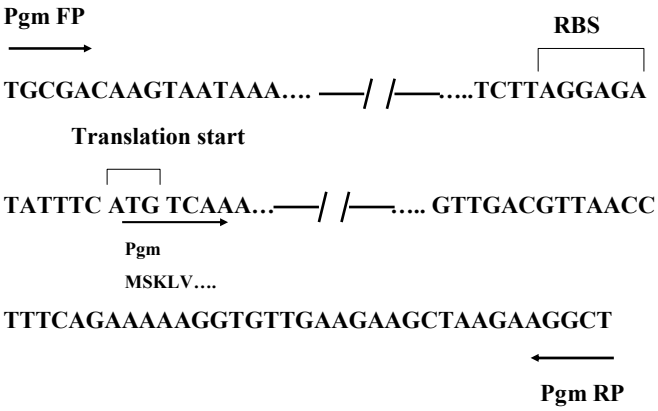


Figure 3.35. Nucleotide sequence of the putative pgm promoter of *Lactobacillus acidophilus* with coding sequence for the first 39 amino acids. Oligonucleotides pgmFP and pgm RP were used to amplify the desired region. The location of the RBS and putative translation start site are indicated on the sequence.

3.7.3. Estimation of IL2 expression or secretion in recombinant LGG

Murine IL2 cDNA was amplified from pBud- IL2 plasmid by PCR using the primers listed Table 2.1 and cloned in frame with secretory signal sequence of slpA gene and the recombinant plasmid was electroporated to LGG. After transformation, LGG Ery+ positive colonies were selected and their growth curves established. Culture supernatant or bacterial pellet from LGG-IL2 (pLP500ldhp-IL2), LGG-IL2 (pLP500slpAp-IL2), and LGG-IL2 (pLP500pgmp-IL2) were harvested after 20 -22 hrs of culture (late exponential phase) for IL2 estimation and LGG-IL2 (pLP500ldh-slpAp-IL2) were harvested at 24 – 26 hrs of culture since it grows a bit slower than the other recombinant LGG. At this time the bacterial CFU was approximately 2 x 10^9 /ml. Protein was extracted from bacterial cell lysate and analyzed for IL2 along with the supernatants. IL2 was 100% secreted in LGG-IL2 (pLP500slpAp-IL2), LGG-IL2 (pLP500ldh-slpAp-IL2) and there was only 20% of IL2 was secreted in LGG-IL2

(pLP500ldhp-IL2). There was no IL2 secretion observed in LGG-IL2 (pLP500pgmp-IL2). IL2 secretion from 2 x 10^9 CFU LGG was about 96 ng or 20 ng under ldh-slpA tandem promoter or slpA promoter respectively and the total production (intracellular and secretory) of IL-2 under ldh promoter was about 1.76 ng (Figure 3.36). Only 1/5 of the total protein produced was secreted which was about 407 pg/ml.

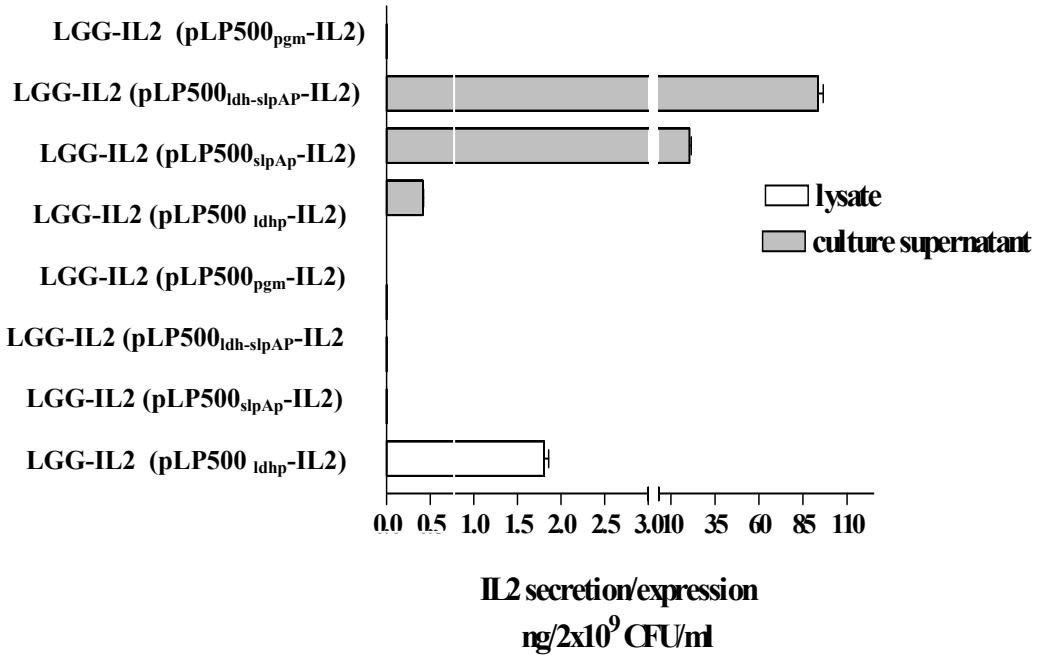


Figure 3.36. IL2 secretion or expression from LGG.
Bacterial lysate or culture supernatants were screened for mouse IL2 and bacterial CFU were also analyzed by quantitative plating.

Summary VI

slpA promoter of *L. acidophilus* was recognized well in LGG and produced a high IL2 production. *ldh* promoter, though it was a strong constitutive promoter in *L. caesei* it gave only 2 ng of IL2 production. So the efficiency of the recognition of the *ldh* promoter region may be host dependent. However when expressed as a tandem promoter with *slpA, ldh* produced a higher level of IL2 which was almost 5 times more than that was produced by slpA promoter alone.

Putative promoter of *L. acidophilus* pgm gene was considered as a strong constitutive promoter and the heterologous protein expression under this promoter in *L. acidophilus* was reported before. But we did not see IL2 secretion under this promoter in LGG. So the pgm promoter may not have been recognized in LGG.

Chapter Four
Discussion

LAB based vaccines are being explored for prophylaxis or treatment for infections or allergic diseases. Because of their GRAS status, they can be used without any reservation as it is unlikely that they could induce any adverse effects except the possibility of septicaemia in severely immuno-compromised recipients (Schlegel *et al.* 1998). Among LAB, *Lactococcus lactis* and *L. plantarum* are widely studied as vaccine delivery vehicles. LGG has rarely been analyzed as a vaccine delivery vehicle. However, the beneficial effects of LGG in cancer has been studied in murine models (Seow *et al.* 2009; Lim *et al.* 2002). Its intrinsic anti-cancer effect; ability to adhere strongly and colonize mucosal sites makes this microbe an attractive candidate for evaluation as an antigen delivery vehicle.

4.1. Oral or nasal co-delivery of IL-2 and an antigen, the green fluorescence protein, by *Lactobacillus rhamnosus* GG results in increased antigen specific humoral immune response with enhanced CD8 and CD4 T cells responses

GFP has been expressed in bacteria to track microbes in the host after immunization. In our study we used GFP as a model antigen and also to track the bacteria in the intestines or lung after oral and nasal immunization respectively. The oral route is the main infection site for many pathogens. Orally administered mucosal vaccines induce secretory IgA production (Mestecky *et al.* 2008) and contain the infections at its initial stage by preventing the replication of pathogens. In this present study, LGG secreting GFP or IL2-GFP as a fusion protein both were shown to be able to elicit both mucosal and systemic humoral responses after oral immunization in mice which was in agreement with a study

conducted by Steidler *et al* (1998). Oral immunization of LGG elicited a sustained mucosal IgA induction and *ex vivo* culture of the intestines also showed a higher IgA level 56 days after the last booster immunization. Intranasal immunization with both modified LGG induced GFP specific serum IgG, IgA, IgM and SIgA. While comparing the immune induction in oral and nasal immunization, nasal immunization produced a higher specific humoral or cellular immune response. The advantage of intranasal over oral route in mucosal immunization has been demonstrated with recombinant *Lactococcus lactis* (Cortes-Perez *et al*. 2007). Intransal immunization with *lactococci* displaying human papillomavirus type 16 (HPV-16) E7 antigen at its surface (LL-E7) and secreting IL12 induced a higher antigen specific IgG or IgA in serum or gastric lavage fluid (GAL) respectively and enhanced E7 antigen specific IFNγ secreting T cells in spleen more than observed in oral immunization. IL2 coexpression with GFP enhanced immune response after both in oral and nasal immunization. The fusion of IL2 to GFP resulted in significantly increased GFP specific serum IgA and IgG compared to LGG-GFP immunization in both oral and nasal immunization. Steidler *et al* (1998) have previously shown the adjuvant effects of cytokines produced by modified *Lactococci* were observed if they were secreted. Several studies also have been done to elucidate the significance of IL2 or IL4 in the induction of B cell proliferation and Ig secretion (Franz *et al.* 1991; Valle *et al.* 1991; Forman *et al.* 1991). Addition of IL2 to cultures of B cells activated with anti Ig or $CD4^+$ T cells results in enhancement of antibody secretion (Croft *et al.* 1991; Boom *et al.* 1988; Abraham *et al.* 1992) and moreover in oral immunization, IL2 is known to stimulate $CD3\epsilon^-$ $IL2R\alpha^+$ cells at the gut lamina propria to secrete IL5 which

helps B cells to produce IgA (Kuraoka *et al.* 2004). IL2 co-expression correlated with a significant accumulation of IgA^+ B cells in NALT and CLN after nasal immunization. Clonal expansion of IgA^+B cells could occur in the NALT prior to the dissemination of sensitized lymphocytes to mucosal and non mucosal lymphoid tissues (Kiyono *et al.* 2004; Hameleers *et al.* 1991; Tilney *et al.* 1971). *Lactobacillus* delivery of GFP (approximately 0.1 ng) resulted in approximately 0.3 or 2.0 μg/ml of GFP specific IgG antibodies being produced from LGG-GFP, while co-delivery of IL2 and GFP resulted in more antibody production namely 0.52 or 3.2 μg/ml of GFP specific antibodies after oral or nasal immunization respectively. In contrast delivery of 10μg of purified Tat protein either alone or with macrophage-activating lipopeptide-2 [MALP-2] or Freund's adjuvant produced 7, 68 and 173 μg/ml respectively of specific IgG antibodies after nasal immunization (Borsutzky *et al*. 2003). This clearly highlights the efficacy of *Lactobacilli* and IL2 as adjuvants and our data indicates that a low level of secreted IL2 is sufficient to produce significant adjuvant effects namely a 1.73 fold (oral) and a 1.6 fold (nasal) increase in antibody production.

The increased level of anti-LGG antibodies was not as great as the increase in GFP antibodies produced by the IL-2 fusion protein. LGG-IL2-GFP induced higher GFP specific serum IgG or sIgA over LGG specific antibodies than LGG-GFP in oral and nasal immunization. Though nasal immunization produced higher GFP specific IgG and IgA, IL2 mediated specific antibody induction (GFP specific Ab over LGG specific Ab) was higher in oral immunization. In oral immunization LGG-IL2-GFP elicited 2.5 or 1.33 fold higher specific IgG or IgA respectively compared to 1.33 or 1.2 folds induced after nasal immunization.

Thus *Lactobacilli* with IL2 co-expression are excellent delivery vehicles as they do not evoke a strong antibody response against themselves. The CD8 recall response was similar to that reported by Poo et al. (Poo *et al.* 2006). After oral immunization with 5 x 10^9 cfu *L. casei* expressing an E7 antigen on the surface they obtained about 500 plus IFN-γ secreting $CD8^+$ T cells in contrast to the 187 ± 31 GFP specific IFN-γ secreting T cells produced with 1 x 10^8cfu of LGG-IL2-GFP immunization in this study. The differences between our studies may be due to the strain difference (Shaw *et al.* 2000; Oliveira *et al.* 2006) and the mode of the delivery of the antigen i.e. surface bound rather than secreted or intracellular expression.

Microarray and semi quantitative PCR analyses showed significantly increased gene expression of CCR2, IP-10 and CD8a in mice lungs immunized with LGG-IL2-GFP. Interferon inducible protein of 10 kDa (IP-10 or CXCL10) is known as one of the potent chemokines that regulate the migration of effector T lymphocytes. Vaccination with DC2.4 cell line transduced with IP-10 gene generated strong E7-specific $CD8^+$ T cell immune responses and mediated a stronger anti-tumor effects against an E7- expressing murine tumor cell (TC-1) (Kang *et al*. 2009). Increased IP-10 expression in LGG-IL2-GFP immunized mice lung was observed with elevated expression of CD8a suggesting that IP-10 would have probably induced the migration of $CD8^+$ T cells. CCR2 expression was reported to be up-regulated following growth factor deprivation and its ligand CCL2 has a role in the proliferation of $CD8^+$ functional T cells in response to IL-2 or to secondary antigenic challenges (Diaz-Guerra *et al.* 2007). However, the underlying mechanism for the concomitant expression of CCR2 not CCL2 with CD8a expression in LGG-IL2-GFP immunized mice lung is not

clear. Nonethless gene expression analyses of the lung suggested LGG-IL2-GFP immunization preferentially increased $CD8^+$T cells.

IL2 was initially described as a negative regulator of the memory $CD8^+$ T cell response since IL2 was reported to cause activation induced cell death (AICD) among T cells (Marks-Konczalik *et al.* 2000) and injection of anti-IL2 antibody decreased the numbers of memory phenotype $CD8^+$ T cells in animals by affecting dividing cells (Ku *et al.* 2000). But, recent reports have demonstrated the positive role of IL2 on memory $CD8^+$ T cells as injection of anti-IL2 antibody increases the biological activity of IL2 possibly through the formation of immune complexes (Boyman *et al.* 2006). In this study it was showed that IL2 secreted at low level (approximately 20 pg) is sufficient to produce significant $CD8^+$ T cell response. The mechanism of enhanced $CD8^+$ T cell response mediated by low level of IL2 was not clear. It is posible that fusion with GFP increases IL-2 survival *in vivo* (half-life normally about 10 mins) and this ensures that though very low levels are expressed it is sufficient to enhance the responses to the antigen (Melder *et al.* 2005; Smith *et al.* 2006). Another possibility is that IL2 physical linkage to antigen may have led to an enhanced MHC- class I presentation of antigen as observed for the E7-IL2 DNA vaccine (Lin *et al.* 2007) which elicited an enhanced $CD8^+$ T cell immune response. Hence, it is therefore postulated that *lactobacilli* stimulation through TLR2 on T cells may further enhance the T cell response by IL2 linkage since TLR2 induced IFNγ production was enhanced in the presence of IL2 (Imanishi *et al.* 2007). In the experiments conducted in the present study, culturable LGG were isolated from MLN or NALT in large numbers 24hrs after oral or nasal immunization respectively. Viable IL2-GFP secreting LGG might engage TLR2

on T cells at MLN or NALT and act in synergy with IL2 (IL2-GFP) to modulate the IFNγ secretion capacity of T cells (Cottalorda *et al.* 2009). More studies will need to be carried out to determine the detailed mechanism that causes the improved $CD8^+$ T cell response.

The M cells located in the FAE are believed to be a route for pathogen invasion into the circulation and for presentation of antigens to the immune system (Neutra *et al.* 1999). The adherence of *lactobacilli* to FAE was observed in a previous study (Edelman *et al.* 2002) and is in line with earlier report of association of certain *lactobacilli* with Peyer's patches of the mouse (Plant *et al*. 2001). However the possible interaction of *lactobacillus* with M cells has not been reported till now. In this study, it was demonstrated for the first time the M cells mediated LGG uptake in intestinal villus. After being transported through M cells, bacteria are engulfed by DC or macrophages in the sub-epithelial dome (SED) region underlying the FAE and transported to MLN (Ruedl *et al.* 1996). Macpherson et al (2004) reported after oral administration of 10^9 *Enterobacter cloacae,* 1000 or 800 CFU bacteria were recovered from MLN at 24 or 48hrs respectively and no bacteria were recovered from splenocytes. In agreement with their observation, there was a high number of live LGG were isolated from MLN in this current study although there were also many live LGG (178 ± 41) isolated from spleen after 24hrs of immunization. However the number of bacteria were reduced after 48hrs (15 ± 4) and completely cleared in 1 week with the oral administration of 1 x 10^8 CFU LGG-IL2-GFP. Their finding with gastric lavage of *E. cloacae* showed that commensal bacteria are prevented from entering the systemic immune compartment like spleen as they are efficiently killed by macrophages. However this present study indicate that

their finding with *E. cloacae* may not be applicable to all commensal bacteria. A previous study showed that LGG are resistant to the intracellular killing activity of macrophages (Asahara *et al.* 2003). Moreover, *L. rhamnosus* and *L. caesei* have been sometimes reported as the causes for infective endocarditis (Harty *et al.* 1994). Though *Lactobacillus* bacteremia was a very uncommon condition, it was believed to occur due to translocation of the bacteria across intestinal mucosa (Antony *et al.* 1996). So the questions are still unanswered whether the LGG-loaded DC are only confined within the mesenteric lymph nodes and whether $CD11c^{-}$ $CD11b^{+}$ intestinal murine macrophages are able to kill LGG effectively *in vivo*. These are avenues for further studies to redefine the compartmentalization of the immune responses to *lactobacilli*.

In summary, the results of this study clearly demonstrate the beneficial effects of low levels of IL2 when expressed as a fusion protein with an antigen and conveyed by *Lactobacilli,* to elicit enhanced local and systemic immune responses to the antigen, as well as the safety and efficacy of Lactobacilli secreting cytokines. It is likely that the immune response to antigens delivered by LGG could be increased by either the delivery of more *Lactobacilli* (Ho *et al.* 2005; Russell *et al.* 1996) or by using plasmids with different promoter strengths so as to increase antigen production **(**Narita *et al.* 2006; Sorvig *et al.* 2005) or even using co-expression of different cytokines.

4.2. *Lactobacilli* secreting IL15/IL2/IL7 and antigen stimulate bone marrow derived dendritic cells and increase antigen specific cytotoxic T lymphocytes responses

There was an improved antigen production by LGG using the slpA promoter and secretory signal and that cytokines especially IL15 enhanced antigen

presentation and activation of T cells. The cytotoxic T cells produced by DCs primed with LGG secreting antigen or cytokine antigen fusion proteins specifically recognized tumour cells secreting PSA.

It was previously reported that the *L. acidophilus* S layer promoter (P-*slpA*) was three times more effective than the most active homologous *L casei* promoter (Boot *et al.* 1996) and it directed efficient transcription in *L rhamnosus* as well (McCracken *et al.* 2000). The S-layer proteins are efficiently secreted as well, so the promoter elements as well as the signal sequence were used to produce LGG that secrete antigens. All the antigens produced were secreted unlike LGG transformed with the PLP500 plasmid with the ldh promoter, where most of the antigen produced remained within the bacteria.

L. reuteri reduced and *L. casei* induced up regulation of CD86 (Christensen *et al.* 2002). LGG stimulated human monocyte derived DCs at a low bacterial dose (5:1 – bacteria : DC ratio) showed only moderate expression of co-stimulatory molecules and produced low levels of cytokines (Veckman *et al.* 2004). However a high dose (100 : 1 – bacteria : DC ratio) of LGG can have stimulatory effects on DC maturation and antigen presentation.

The present study showed LGG stimulation alone on BMDC produced high levels of IL-12 without CD40L help. IL12 production was also induced in DC by stimulation with *Toxoplasma gondii* extract or lipopolysaccharide without CD40L help (Reis e Sousa *et al.* 1997). CD40L has been known to synergize with soluble tachyzoite antigen (STAg) of *T. gondii* or CpG DNA to induce high levels of IL-12 production by DC (Schulz *et al.* 2000). DC from IL15 knock out mice ($IL15^{-/-}$) are known to produce low IL-12p70 and

exogenous supply of recombinant IL15 will increase IL-12p70 production but not in IL15R$^{-/-}$ DC (Ohteki *et al.* 2001). In agreement with these findings BMDC stimulated with LGG-S-IL15-PSA produced a significantly higher amount of IL12p70.

T cells incubated with LGG-S-IL15-PSA treated DC showed high IFNγ production and CTL response on target cells indicating efficient antigen presentation to T cells. IL-15 itself is able to activate DC *in vivo* and *in vitro*, to enhance the ability of DC to stimulate Ag-specific CD8^{+} T cells (Ohteki *et al.* 2001; Pulendran *et al.* 2004; Mattei *et al.* 2001). The mechanism by which IL15 mediates enhanced antigen presentation has not been defined but evidence in the literature points to several possible pathways. Firstly, enhanced antigen presentation could be a consequence of increased IL12 production engendered by LGG-S-IL15-PSA treatment (Christensen *et al.* 2002). The second possible mechanism for increased antigen specific CD8 $^{+}$ T cells expansion could be through trans presentation of IL15 by DC. IL15 can up regulate IL-15αR expression on DC and IL15αR can efficiently trans present the bacteria secreted IL15 to IL-2Rβ/γ_c heterodimeric receptor on responding cells to initiate signaling (Wu *et al.* 2008) which up-regulates anti-apoptotic signals such as Bcl-2, promoting T cell survival. The IL15/IL15R complex can recycle through the endosomal vesicles for several days (Dubois *et al.* 2002) and so the signalling activity would persist even after removal of the bacteria by gentamycin treatment. No evidence exists for such a mechanism for IL7, but stimulated DC do produce IL7. Expression of IL7R on B or T cells lineage was reported (Sudo *et al.* 1993) but its expression on DC has not been defined yet.

Another possible mechanism is the effect of IL15 on the regulation of the antigen processing machinery (APM) in DC. Tumour cells down regulate APM expression in DC and recombinant IL15 administration restores MHC class I APM component expression in DC (Tourkova *et al.* 2005). Trauma has also been shown to suppress splenic DC antigen presentation and exogenous IL15 can attenuate this response. (Kawasaki *et al.* 2009).

PSA specific cytotoxic effect on target cells in T cell presentation with LGG-S-IL2-PSA stimulated DC was not significantly different with LGG-S-PSA stimulation. IL2 role on DC has not been clearly defined as yet. DC from IL-$2^{-/-}$ mice are impaired in their ability to induce allogeneic $CD4^+$ T-cell proliferation (Granucci *et al.* 2001). DC treated with IL2Rα antibody and LPS caused a reduction in IL-12, IL-1, TNF-α, IL-6, and IFN-γ production and decreased the DC ability to prime allogeneic $CD4^+$ T cells compared with stimulation with LPS alone (Mnasria *et al.* 2008). Hence, LGG-S-IL2-PSA treated DC may have a role in inducing $CD4^+$ not $CD8^+$ T cells.

T cells incubated with LGG-S-IL7-PSA treated DC showed CTL response on target cells which is significantly different from LGG-S or LGG-S-PSA treatment. High IL7 was reported to promote not $CD4^+$ but $CD8^+$ T cell proliferation and IL-7 signalling down regulates MHCII expression in $IL7R\alpha^+$ DC that contribute to diminished $CD4^+$T cell homeostatic proliferation (Guimond *et al*. 2009). Further study may be required to investigate whether IL7 secreted by LGG-S is enough to induce the preferential proliferation of $CD8^+$ T cells which would be observed as higher cytotoxic effect on target cells.

In summary, the data in this study indicate that LGG as a promising antigen delivery vehicle and that IL15 or IL7 is a good vaccine adjuvant.

4.3. Cross talk between LGG treated neutrophils and dendritic cells and its effect on DC activation and antigen presentation

In agreement with previous reports on microbes treatment with neutrophils (Bennouna *et al.* 2003), LGG treatment with neutrophils did not produce a vey high bioactive IL12 rather it produces a high level of IL10 and TNFα. Moreover, LGG treated neutrophils did not induce any of the co-stimulatory molecules or MHC II expression, but only showed elevated expression of the MHC I molecules. Human polymorphonuclear neutrophils (PMN) require prior treatment of IFNγ, GM-CSF and IL3 to express MHC II molecules (Gosselin *et al.* 1993). Even after IFNγ and GM-CSF treatment, not all antigens were known to be processed and elicit specific T cells proliferation presented in a MHC II restricted manner. Super antigen but not tetanus toxoid (TT) treated PMN stimulated specific T cell proliferation (Fanger *et al.* 1997; Radsak *et al.* 2000). In mice, neutrophils purified from peritoneal exudate cells (PEC) were shown to express MHC II molecules expression and neutrophils pulsed with $OVA_{323-339}$ peptide induced specific $CD4^{+}$T cells proliferation (Culshaw *et al.* 2008). However, we did not observe increased MHC II expression in bone marrow derived neutrophils (BMN) after LGG treatment. It may suggest bone marrow derived neutrophils may have a different MHC expression profile compared to PEC neutrophils. Potter *et al* (2001) reported neutrophils process the exogenous bacteria in alternate MHC I pathway and present the antigen to T cells. It was presumed that following phagocytosis, LGG also would be processed in the

alternate MHC I pathway since it elicited only the expression of MHC I molecules. After antigen presentation for 2 hours neutrophils elicited 1.05 or 1.49 ng of IFNγ which are slightly higher than 0.70 or 1.11 ng produced in overnight stimulation with LGG-S or LGG-S-IL15-PSA treatment respectively. The reduced IFNγ production in overnight stimulated neutrophils suggests the suppressive effect rendered by higher IL10 production (Fiorentino *et al.* 1991). Increased expression of co-stimulatory molecules on DC in neutrophils-DC co-culture clearly showed bacteria treated neutrophils could deliver the maturation signals to immature DC. However, LGG treated neutrophils stimulate higher IL10 but not IL12 from DC which is an opposite effect seen in BCG (Morel *et al.* 2008) or Toxoplasma (Bennouna *et al.* 2003) treatment with neutrophils. High IL10 along with increased production of TGFβ in DC-LGG treated neutrophils co-culture generates an anti-inflammatory milieu. IL10 produced in neutrophils-DC co-culture was positively correlated with the concentration of the bacteria used to stimulate neutrophils (data was not shown). After encountering a high number of bacteria, neutrophils may undergo apoptosis, (Ocana *et al.* 2008) perhaps TRAIL/Apo-2L mediated as seen in BCG stimulation (Kemp *et al.* 2005) and apoptotic neutrophils may be phagocytosed by DC (Stark *et al.* 2005). The uptake of apoptotic cells by phagocytosis could trigger a powerful anti inflammatory signal (Savill *et al.* 2000; Stuart *et al.* 2002) like increased release of IL10 (Steinman *et al.* 2000), TGF-β and inhibition of IL12 and IL8 (Voll *et al.* 1997; Kim *et al.* 2004). Apoptotic neutrophils bind to protein S in serum which interact with TAM receptor (protein tyrosine kinases-TYRO3, AXL and MER) (Lemke *et al.* 2008) expressed on DC or macrophage (McColl *et al.* 2009) that augment phagocytic

removal of apoptotic neutrophils. Further investigation is required to examine whether the apoptosis would be induced in neutrophils with LGG stimulation (1:100 neutrophils: LGG ratio) and the level of TAM receptor expression on DC in DC – neutrophils (stimulated with LGG) co-culture.

The immunosuppressive environment observed in neutrophils-DC co-culture did not affect the T cell presentation that was characterized by high IL2 and IFNγ which were almost the same level as the DC or much higher than neutrophils mediated direct antigen presentation. Recombinant LGG treated neutrophils provided antigen specificity to DC when it was co-cultured with DC and also rendered a moderate cytotoxic effect in T cell presentation. This ensures the efficacy of LGG based antigen delivery in inducing immune response through neutrophils alone in the absence of direct bacteria-DC encounter. Further study may be required to analyze the proportionate antigen specific T cells generated in neutrophils mediated direct or indirect antigen presentation and the inhibitory mechanism of inflammatory cytokines production in DC that phagocytose the apoptotic neutrophils.

In summary, the present study demonstrates the effect of LGG in neutrophils- dendritic cells cross talk, highlights the balancing effect of LGG treated neutrophils on DC to shut down the inflammatory response. However further studies may be required to demonstrate the LGG treated neutrophils-DC cross talk *in vivo* in the priming of antigen specific T cells.

4.4. Improvement of antigen production in LGG using ldh-slpA tandem promoter

For high protein production, a strong promoter is necessary. Improvement by genetic engineering to induce high protein production in the target strain have been carried out by modifying the promoter sequence (Estrem *et al*. 1998; Jensen and Hammer *et al*. 1998; Solem and Jensen *et al.* 2002). Protein production was shown to have increased in recombinant bacteria under two or three tandem promoters in contrast to one alone in *Bacillus subtilis.* (Widner *et al*. 2000). This strategy has been applied in *Lactococcus lactis* for the expression of staphylokinase variant gene (*sakXH*) under the control of two tandem promoters (P32-P*lacA*) and showed improved protein production (Wei *et al*. 2002). In our study we developed a protein expression system using LGG as the host strain and constructing two tandem promoters, ldh and slpA for protein expression. The expression of α amylase was improved under the control of ldh promoter that was integrated with the untranslated leader sequence (UTLS) of the slpA gene from *L. acidophilus* (Narita *et al*. 2006). The structure of the UTLS is deduced to be important in the mRNA stability (Daguer *et al.* 2005; Fournier *et al.* 2001) and in our study we used the whole promoter sequence of the slpA gene including UTLS coupled with ldh core promoter. The RBS of the slpA promoter was used in this study instead of the RBS of the ldh to prevent the destruction of the structure. The fusion expression system increased the IL2 production to almost 5 folds compared to IL2 secreted under slpA promoter alone.

In another strategy to improve the IL2 secretion we used a putative promoter of pgm and its secretory signal sequence including coding sequence

for 39 aminoacids for pgm protein to generate pgm-IL2 fusion protein. Phosphoglycerate mutase (PGM), an important enzyme in the glycolytic pathway, catalyzes the transfer of a phosphate group between the 2 and the 3 positions of glyceric acid. *Lactobacilli* which grow better in anaerobic environment (Pant *et al.* 1996) may have a high expression of pgm. Mohamadzadeh et al reported the oral administration of *L. acidophilus* mediated delivery of *Bacillus anthracis* protective antigen-dendritic cells targeting fusion protein (PA-DC pep) under the expression of *L. acidophilus* pgm promoter generated protective immunity against *B. anthracis* lethal challenge. Though we followed the same strategy, the transformed LGG did not result in IL2 production. We surmise that the pgm promoter may not have been recognized in LGG.

4.5. Conclusion

In most of the studies in which *Lactobacilli* have been explored as vaccine candidate in either oral or nasal route, bacteria used for immunization were more than 10^8 CFU. In the present study 10^8 CFU LGG was used and demonstrated the antigen specific humoral and cellular immune induction. Despite the low IL2 level expressed in LGG as IL2-GFP fusion protein, it convincingly enhanced GFP specific immune induction. Strikingly, the enhanced immune induction has lasted even 50 days after the booster immunization. While comparing oral or nasal immunization, nasal immunization showed better immune response. Live bacterial administration were mostly discouraged in nasal immunization as live bacteria may cause lung infection and some times may result in pneumonia. However because of the GRAS status *Lactobacilli* administration did not

produce any lung damage except scattered cellular infiltration observed after few days of immunization.

L. acidophilus slpA promoter enhanced the protein expression more than 10 folds in LGG. Moreover slpA secretory signal was well recognized in LGG and showed almost 100% extracellular delivery of heterologous protein. Protein expression under SlpA-ldh tandem promoter improved the protein expression to 5 or 50 folds more than the protein expression under individual slpA or ldh promoter respectively. IL15 co-expression with tumour antigen PSA enhanced antigen presentation and showed a higher antigen specific cytotoxic effect followed by IL7 co-expression. It also demonstrated an enhanced DC mediated antigen presentation to T cells and induced a high $CD8^+$ T cell proliferation. Recombinant LGG treated neutrophils interact positively with DC to produce T cell presentation and antigen specific cytotoxic effect though it has not been as high as direct DC antigen presentation. However it needs further investigation to characterize the anti-inflammatory immune induction in DC-neutrophils mediated T cell presentation.

Seow et al (2009) reported that intravesical administration of LGG recruited large numbers of neutrophils and macrophages to the tumor site and showed better cure rate than BCG (89% vs 77%) which is considered as gold standard for bladder cancer immunotherapy. This cytotoxic effect may be enhanced further with the intravesical instillation of recombinant LGG that express specific tumour antigens like BLCA-4 (Van Le *et al*. 2004) since recombinant LGG can induce neutrophils mediated antigen specific cytotoxic effect.

4.6. Future directions

It has been shown convincingly in this series of experiments that LGG could deliver the antigen efficiently and induce specific immune response. The beneficial effect of cytokine-antigen co-expression in enhancing antigen specific cytotoxic effect has been also demonstrated. However, the enhanced antigen presentation and cytotoxic effect in common gamma chain cytokine – antigen expressing LGG treated DC needs to be shown *in vivo* and the mechanism for the enhanced immune response after immunization with fusion proteins also needs to be unravelled. As such further research should be aimed at the following:

1. Future experiments should determine how IL2 in pg levels preferentially produces a high $CD8^+$ T cell response. It may be extended to see the effect of high IL2 (in ng to μg level) in the induction of $CD8^+$T cell response.

2. Though the cellular infiltration observed in 35^{th} day of post primary nasal immunization was reduced on 80^{th} day, some inflammatory cells were still present and thus it may be required to examine the infiltration or inflammatory changes up to 100-180 days to rule out LGG mediated persistent or chronic lung inflammation. If the latter is present than LGG vaccines should only be delivered via the oral route.

3. LGG-S-IL15-PSA treated DC showed high IFNγ production and CTL response on PSA secreting tumour cells indicating efficient antigen presentation to T cells *in vitro*. In future experiments, IL-15 mediated enhanced antigen presentation has to be confirmed *in vivo* by analyzing whether mucosal immunization of IL15-antigen fusion protein secreting LGG efficiently kill the

tumour cells compared to immunization with antigen alone or with other cytokine-antigen fusion protein in an animal tumour model.

4. Future experiments may also be focussed on neutrophil mediated *in vivo* indirect antigen presentation to T cells and the analysis of T cell mediated antigen specific cytotoxic effect in the murine bladder cancer model by intravesical instillation of recombinant LGG.

5. In neutrophils-DC cross talk study, DC mediated antigen presentation to T cells through neutrophils engenders an anti-inflammatory milieu which was represented by a significantly higher TGFβ and IL10. Future study may be focussed on the T cell phenotype which secrete TGFβ.

6. Increased antigen production may be imperative for inducing a high antigen specific immune response. Future studies should explore the possibilities of a) stronger promoters and b) to design the cassette to express the antigen and cytokine under different promoters, so that their expression will be manipulated individually to study the impact of cytokine concentration on the immune response.

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