

# **An escherichia coli vaccine co-expressing listeriolysin-O and tumour antigen in cancer immunotherapy and the mechanisms of immune regulation**

Dai, Ming-Shen

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**An *Escherichia coli* Vaccine co-Expressing  
Listeriolysin-O and Tumour Antigen in Cancer  
Immunotherapy and the Mechanisms of Immune  
Regulation**

**A thesis submitted for the Degree of Doctor of Philosophy at  
Queen Mary University of London**

**June 2009**

**Ming-Shen Dai**

**Supervisor: Professor Nick Lemoine**

**Centre of Molecular Oncology, Institute of Cancer  
John Vane Science Centre, Charterhouse Square  
Barts and The London School of Medicine and Dentistry  
Queen Mary University of London**



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## LIST OF ABBREVIATIONS

AGT	Alternative gene therapy
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BMDCs	Bone marrow-derived dendritic cells
CDC	Cholesterol-dependent cytolysin
CpG	Cytosine-phosphate-guanine
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence Activated Cell Sorter
FDA	Food and Drug Administration
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HTL	Helper T lymphocyte
IPTG	Isopropyl $\beta$ -D-thiogalactopyranoside
LLO	Listeriolysin-O
LPA	Lymphocyte proliferation assay
LPS	Lipo-polysaccharide
MHC	Major histocompatibility complex
OVA	Chicken ovalbumin antigen
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed Cell Death-1
pWT	WT1 peptide
S.C.	Subcutaneous
STAT	Signal transduction and activator of transcription
TAA	Tumour-associated antigen
TSA	Tumour-specific antigen
Tconv	Conventional T cells (CD4 <sup>+</sup> CD25 <sup>-/low</sup> )
TGF- $\beta$	Transforming growth factor- $\beta$
TLR	Toll-like receptor
Treg	Regulatory T cell (CD4 <sup>+</sup> CD25 <sup>+/high</sup> )
WT1	Wilms' tumour gene 1

## ABSTRACT

Some recombinant bacterial strains have been shown to be very efficient in experimental therapies against cancer in rodent models. Amongst them is an *E. coli* that expresses the Listeriolysin-O protein (LLO) and a model tumour antigen ovalbumin (OVA). I have demonstrated the efficacy of *E. coli*-LLO/OVA in preventive or therapeutic models against OVA-expressing tumours. This effect is mediated by specific cytotoxic T-lymphocytes (CTL) against OVA antigen and inhibition of the suppressive function of Foxp3+ T-regulatory (Treg) cells. When applying a “real” and clinically-relevant tumour antigen, Wilms’ tumour-1 antigen (WT1), this vaccine (*E. coli*-LLO/WT1) is capable of inducing an anti-tumour effect. Furthermore, we have characterised the immunodominant epitope involved in *E. coli*-LLO/WT1-immunisation (*pWT*<sub>130-138</sub>, NPYLPSC<sub>L</sub>) through screening of a peptide library of the WT1 protein by cytokine ELISpot, lymphocyte stimulation effects, MHC stability, and specific cytotoxicity. Also, the effect on Treg when applying a real tumour antigen is still preserved. Co-injection of *pWT*<sub>130-138</sub> with *E. coli*-LLO resulted in an anti-tumour effect equivalent to that obtained with *E. coli*-LLO/WT1, demonstrating that the adjuvant properties of the *E. coli*-LLO vaccine can be exploited in conjunction with peptides.

Treg are recognised as playing important roles in immunotherapy. An ideal vaccine for cancer would stimulate specific cytotoxic responses and suppress Treg function. This study showed that *E. coli*-LLO vaccine suppresses Treg cell function and the Treg RNA microarray analysis revealed expression differences of some cytokine/chemokine genes which could be relevant to the reversal of Treg suppression. This may have important implications for developing anti-tumour vaccine strategies in humans. Overall, this study demonstrated that an *E. coli*-LLO vaccine is effective in cancer immunotherapy, either co-expressed with a real tumour antigen or co-injected with a peptide. The efficacy of this vaccine was due to its ability to dampen Treg suppressive function.

## DECLARATION OF ORIGINALITY

I hereby declare that all the work contained in this thesis is the result of my own independent investigations.

This work has not already been accepted for any degree, nor is it being concurrently submitted for any other degree.

A handwritten signature in black ink that reads "Ming-Shen Dai". The signature is written in a cursive style with a large initial 'M' and a long, sweeping underline.

Ming-Shen Dai

## **COLLABORATORS**

### **Dr. Georges Vassaux and Dr. Nicolas Ferry**

INSERM, CIC-04, Biothérapies hépatiques, CHU Hotel Dieu, Nantes, France.

### **Prof. Hans J Stauss**

University College London, Hampstead Campus, Royal Free Hospital, London,  
United Kingdom.

### **Prof. Tsu-Yi Chao**

Div. of Haematology/Oncology, Tri-Service General Hospital, Taipei, Taiwan  
National Health Research Institute, Taiwan

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Josianne Nitcheu-Tefit, Ming-Shen Dai, Rebecca J. Critchley-Thorne,  
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## **SPECIAL NOTE**

During the period of my PhD study, my father-in-law passed away because of hepatocellular carcinoma with rapid progression. His death had a huge impact on my perception of this type of research and its potential application. Not only the painfulness of losing a family member, but this also truly reflected the hurdles to combat cancer and the necessities to improve the cancer patient survival and life quality. In my profession as a clinical oncologist, this strengthened even further my faith to devote myself to cancer research. I am pretty sure that this is my destiny.



# CHAPTER 1. INTRODUCTION

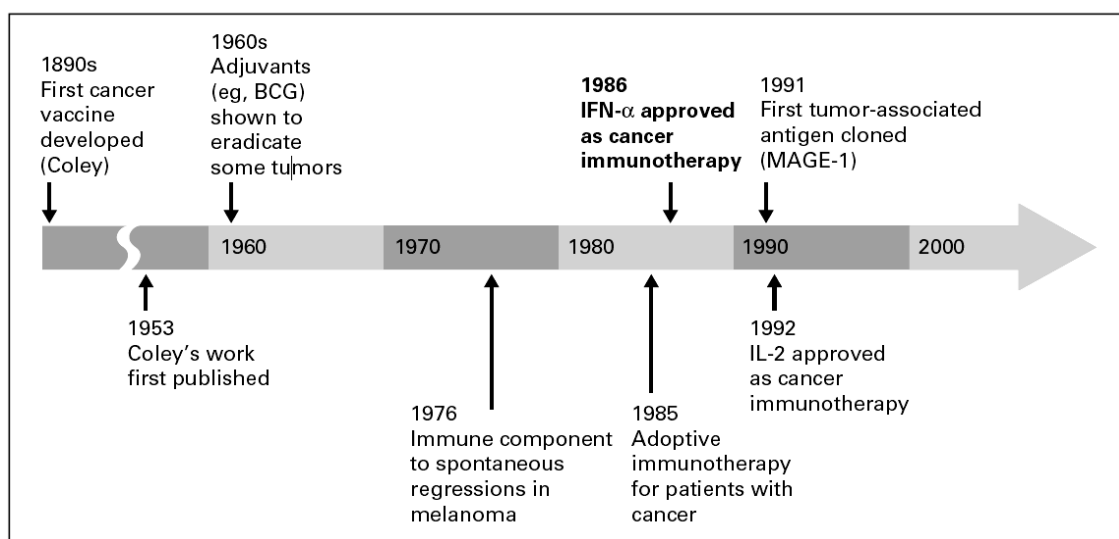
## 1.1 CANCER IMMUNOTHERAPY

### 1.1.1 Emergence of immunotherapy in cancer

Cancer is the second leading cause of death in the industrialised world and the first in Taiwan. It is thought to be the result of several events, including genetic predisposition, transformation by viruses or environmental mutagens such as radiation and chemicals, and tumour promoters leading to abnormal and uncontrolled cell growth. Conventional cancer treatment comprises surgery, radiation, and chemotherapy. The primary tumour can, when diagnosed at early stages, be efficiently treated by a combination of these modalities, preventing the subsequent metastatic spread of the disease. However, these tumours can relapse and tumour-free survival is not always obtained. Despite enormous advances in the development of new chemotherapeutic drugs and improvements in radiotherapy, conventional cancer therapy often falls short of the goal of controlling tumour progression. The development of tumour cell resistance to chemotherapy and to radiation therapy, as well as the toxicity of these treatment modalities, limit the success of the treatment and necessitate the search for better treatment options or new combinations.

The potential of cancer immunotherapy was first introduced by William Coley in 1890, when bacterial products (Coley's toxins) were administered for advanced inoperable cancers, with sometimes dramatic responses (Figure 1) (Nauts, Fowler et al. 1953). After the successful application of *Bacillus Calmette-Guérin* (BCG) vaccine to prevent *Mycobacterium tuberculosis* infection in 1924 (Levine 1947), the possible use of BCG as cancer therapy began from the late 1950s (Old, Clarke et al. 1959). However, the potential of cancer immunotherapy was generally ignored until the latter

part of the 20<sup>th</sup> century, when studies of chemically-induced tumours of inbred mice demonstrated transplantation resistance (Nathanson 1976), and spontaneous regression of melanoma fuelled speculation that immunologic responses contributed to tumour regression. In the 1980s, lymphocytes activated with lectins or interleukin-2 (IL-2) were demonstrated to target tumour cells *in vitro* (Grimm, Mazumder et al. 1982) and exogenous cytokines were investigated in large-scale clinical trials for metastatic melanoma (Kirkwood, Ernstoff et al. 1985).



**Figure 1. Key events in the history of cancer immunotherapy.** BCG, Bacillus Calmette-Guérin; IFN- $\alpha$ , interferon alfa; IL-2, interleukin-2. (Kirkwood, Ernstoff et al. 1985)

The immune system continually recognises and eliminates tumour cells. Tumour-specific lymphocytes can be found in the blood, draining lymph nodes, and the tissues surrounding tumours in patients with actively growing tumours. However, this interaction between tumour and immunity appeared to be considerably more complicated than the early “immune surveillance” theories suggested, mainly because of the need for the immune system to avoid aberrant responses directed at the normal tissues (Pardoll 1998). Cancer recurrence or metastatic spreading usually results from tumour evading immune surveillance and growing too large for the

immune system to eradicate (Dunn, Old et al. 2004). Tumours vary greatly in their immunogenicity and can evade immune elimination even if tumours can be recognised by the host immune system. Therefore, cancer immunotherapy is a promising approach, due to its potential for the eradication of disseminated tumour cells present in the blood circulation, residual cancer cells from primary treatment and micro-metastases in distant organs. The main strategies of cancer immunotherapy, either actively or passively, aim to exploit the therapeutic potential of tumour-specific antibodies and cellular immune effector mechanisms. A better understanding of both the molecular mechanisms that govern the generation of an effective immune response, and the biology of a tumour, has contributed to substantial progress in the field.

### **1.1.2 Targeting Tumour Antigens**

From the earliest days in the field of tumour immunology, researchers are continuously attempting to discover tumour antigens, which can be targets of immunotherapy, in various kinds of cancer. Tumour antigens can broadly be separated into two groups: tumour-specific antigens (TSA) and tumour-associated antigens (TAA).

TSA, which are usually mutant proteins, are expressed exclusively by cancer cells and are often crucial for tumourigenicity. For example, *Ras* mutations are prevalent in many types of adenocarcinomas including pancreatic (~90%), colorectal (~50%), lung (~30%), and thyroid cancers (~50%) (Hruban, van Mansfeld et al. 1993), and three *Ki-RAS* point mutations (single amino acid substitutions) are found in about 95% of all patients with pancreatic cancers. They are ideal targets for anti-cancer therapy. TSAs are ideal targets for cancer immunotherapy because they are exclusively

expressed by the cancer cell and not in non-malignant tissues, minimising the risk of autoimmune destruction. In addition, because the immune system has not been previously exposed to these antigens, there is no neonatal or peripheral tolerance to these antigens prior to tumour development. During tumour development, the immune system can recognise these determinants as non-self and generate specific, high-affinity antibodies and T cells against them. Another major advantage of tumour-specific antigens is that many of the mutant proteins that result in tumour-specific antigens are themselves essential for tumourigenesis. Several studies have reinforced the importance of targeting such essential proteins with cancer immunotherapy (Lennerz, Fatho et al. 2005). Experimental and clinical observations suggest that those proteins required for the maintenance of the malignant phenotype are less likely to be lost during tumour progression, even under the selective pressure of anti-cancer treatments such as immunotherapy (Sensi, Nicolini et al. 2005). However, targeting TSA would require therapeutic strategies to be tailored to individual patients or small subgroups of patients, making the targeting of TSA more technically challenging and labour-intensive. As a result, the focus of targeting peptides has been gradually moved toward normal molecules that are not tumour-specific but expressed on large groups of cancers, TAA.

TAA represent a group of normal non-mutant molecules that can be subdivided into four major categories according to expression pattern (Novellino, Castelli et al. 2005; Schietinger, Philip et al. 2008):

- Oncospermatogonial antigens (cancer-testis antigens) are expressed by cancer cells but are normally found on spermatocytes/spermatogonia (MAGE, GAGE, BAGE and NY-ESO-1) (Fijak and Meinhardt 2006). These

antigens, like all other tumour-associated antigens, also induce some level of central or peripheral tolerance.

- Differentiation antigens are molecules expressed on non-malignant cells of the same cell lineage as the tumour (TRP-1, gp100, MART-1, tyrosinase, CD20 and EpCAM) (Kawakami, Robbins et al. 1998; Armstrong and Eck 2003).
- Oncofetal antigens are antigens found in embryonic and fetal tissues as well as certain cancers (alpha fetoprotein, carcinoembryonic antigen, Wilms' tumour-1, and 5T4) (Coggin, Barsoum et al. 2005).
- Over-expressed antigens are normal proteins whose expression is up-regulated in cancer cells (PSA, wild-type p53, Her2/Neu, Telomerase, and EGFR) (Novellino, Castelli et al. 2005).

Because of their expression in normal, non-malignant tissue, TAA are more likely to have induced immunologic tolerance and are less likely to stimulate effective immune responses (Cloosen, Arnold et al. 2007). Self-reactive T cells are readily deleted and/or functionally inactivated, and when not deleted, have a reduced capacity to recognise target antigens (Yu, Theoret et al. 2004). If a cancer vaccine does break tolerance to a tumour-associated, shared antigen, with emergence of self-reactive T cells, destruction of normal tissue or even fatal autoimmune damage can result (Ludewig, Ochsenbein et al. 2000; Gilboa 2001). Although T cells can be engineered to express receptors with high (nanomolar) affinity for self-antigens that are highly expressed in cancers, there is concern that such T cells could cause significant autoimmune damage if used therapeutically. Such autoimmune damage may be tolerable if the self-antigen is expressed only in non-essential, normal tissue. It is unclear, however, whether T cells that target self-antigens in tumours could be

similarly effective, even if normal cells expressing those antigens were dispensable. Another concern is that targeting of TAA that are non-essential can lead to the emergence of antigen loss variants (Lozupone, Rivoltini et al. 2003). In summary, selection of TSA or TAA should be judged according to the biologic nature of the target tumour, the immunotherapeutic strategies undertaken, and of course the risks/benefits.

### **1.1.3 T cells and Antigen Recognition**

The immune response is broadly classified into either the innate, antigen-nonspecific response, or the adaptive, antigen-specific response. Leukocytes of the innate immune system reside in peripheral tissues and circulate through the blood and secondary lymphoid tissues (the spleen and the lymph nodes), acting as immunologic sentinels for detecting external pathogens. Similarly, B and T lymphocytes traverse the body to mediate the adaptive immune response. These cells express a comprehensive repertoire of antigen-specific receptors (cell surface immunoglobulin receptors for B cells, and cell surface T cell receptors (TCR) for T cells) that can recognise over one million distinct antigens (Oltz 2001). Whereas the B cell antigen receptor directly binds to antigenic determinants present on soluble proteins, carbohydrates, or nucleic acids, the T cell antigen receptor binds most commonly to short fragments of antigens that have been broken down and loaded onto Major Histocompatibility Complex (MHC) molecules. Thus, B cells can see antigen directly, and respond by differentiating into immunoglobulin-secreting plasma cells. In contrast, T cells detect processed antigen in the context of self MHC molecules, thereby providing a basis for self-nonself discrimination (Germain 1995). Two major subsets of T cells collaborate to mediate an effective immune response.

CD4<sup>+</sup> helper T cells are activated after binding peptide antigen presented by MHC Class II molecules, and provide cytokine-mediated “help” both to shape the B cell-mediated humoral response, and to maximise the quality and durability of the CD8<sup>+</sup> T cell-mediated cytotoxic T lymphocyte (CTL) response (Pulendran 2004). CD4<sup>+</sup> T cells can be further divided into T helper type 1 cells, which secrete interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) to promote CTL activity, and T helper type 2 cells, which secrete interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-6 (IL-6), and promote humoral and allergic immune responses. Of these two T helper phenotypes, the T helper type 1 phenotype is generally considered to contribute more to antitumour immunity (Kidd 2003). CTLs are activated after TCR binding antigen presented by MHC Class I molecules, deploying a payload of cytokines and enzymes that can effectively lyse diseased cellular targets. Thus, it is CD8<sup>+</sup> effector T cells that are critical for destroying host cells altered by either viral infection or oncogenic transformation. In order for CD8<sup>+</sup> CTL to recognise and destroy diseased cellular targets, they must migrate throughout the body to interact with professional antigen presenting cells (APCs) (usually DCs, B cells, macrophages, and  $\gamma\delta$ T cells) (Trombetta and Mellman 2005). After the priming APC-T cell interaction, CD8<sup>+</sup> CTL subsequently engage target cells that have been altered by pathogens or neoplastic transformation.

T cell activation is initiated at the molecular level by the TCR-mediated recognition of antigenic epitopes bound to MHC molecules. The specificity of most T cell responses is conferred by the  $\alpha\beta$  TCR. These TCRs are formed by two transmembrane glycoproteins, each composed of one extracellular variable, which determines the diversity of TCR, and constant domain joined by a hinge region to the transmembrane domain. The presence of MHC diversity also impacts antigen

recognition by T cells indirectly by controlling peptide binding to MHC, and directly by physical contacts between the TCR and MHC molecule (Davis, Boniface et al. 1998). The cellular interactions during the priming and effector cross-talk will affect the ultimate quality and character of the immune response, reflected by the size, phenotype (effector, tolerogenic, or memory), and functional status (cytokine secreting, cytotoxic) of the activated T cell repertoire. Due both to their delicate antigenic specificity and their capacity for vigorous secondary response, the use of T cells for the immune-mediated therapy of cancer has attracted great interest. With the advances in understanding how T cells see antigen at the molecular and cellular level, many preclinical and clinical studies in T cell-based immunotherapy are undergoing. Future scientific progress elucidating the mechanisms controlling antitumour T cell activity will provide the foundation for developing cancer immunotherapy strategies that harness the power of the antitumour T cell response.

#### **1.1.4 Different strategies of cancer immunotherapy**

The first cancer vaccines were composed of irradiated or otherwise inactivated whole tumour cells (Ward, Casey et al. 2002), based on the success of using attenuated pathogen vaccines in infectious diseases. This immunisation strategy was successful in mouse models and produced tumour-specific immune responses and rejection of a tumour-challenge. Concerning the safety and immunogenicity of whole tumour cells, and also after deciphering the exact mechanism of antigen presentation and T-cell activation by the immunologists, several different immunisation strategies, aimed at specific target protein/gene in tumour or using different adjuvant to enhance immune reactions or modify antigen presentation, have been introduced in past ten years.



#### **1.1.4.1 BCG vaccine**

The possible use of BCG as cancer therapy began with landmark studies in the late 1950s by Lloyd Old at the Sloan-Kettering Institute in New York (Old, Clarke et al. 1959). In a series of elegant experiments Old demonstrated that mice infected with BCG showed increased resistance to challenge with transplantable tumours. The action of BCG was attributed to the general augmentation of immunological reactivity. BCG was found to activate macrophages, which can inhibit or destroy cancer cells and induce necrosis of mouse tumours, providing the first direct evidence that BCG had anti-tumour effects.

In the 1970s Burton et al further demonstrated the tumour inhibitory properties of BCG injected into animals (Zbar, Bernstein et al. 1971). Animals that rejected tumour cells at the site of an infection with BCG showed suppressed tumour growth and delayed hypersensitivity reactions to a subsequent challenge of tumour cells. The clinical use of BCG as cancer therapy began in 1969 when Mathe in France reported encouraging results with BCG as adjuvant therapy for acute lymphoblastic leukaemia, and in 1970 Morton in the United States observed regression of malignant melanoma treated with intralesional BCG. In 1975 deKernion et al reported that isolated melanoma in the bladder was successfully treated with cystoscopic injection of BCG vaccine (deKernion, Golub et al. 1975) . These reports created enormous interest in BCG, hailed as an effective anticancer agent, and clinical trials flourished using BCG against lung, prostate, colon and kidney cancers. Their promise was not fulfilled, however, and BCG was soon replaced by more effective therapies. The only notable exception is bladder cancer, for which remains a standard intravesical therapy against high-grade non-invasive disease. Nowadays, BCG is frequently used as

vaccine adjuvant to enhance immune responses in experimental models (Cox and Coulter 1999; Suttman, Jacobsen et al. 2004).

#### **1.1.4.2 Recombinant Cytokines**

Interferon alpha (IFN- $\alpha$ ) was the first exogenous cytokine to demonstrate anti-tumour activity in advanced melanoma. IFN-2 $\beta$  (Intron A; Schering Corporation), a type I IFN, is a highly pleiotropic cytokine with immunoregulatory, antiproliferative, differentiation-inducing, apoptotic, and antiangiogenic properties in multiple malignancies, and objective tumour response rates of approximately 20% were observed in phase I/II trials for metastatic disease (Kirkwood 2002). However, tolerability is an issue with this regimen, because of flu-like symptoms, anorexia, fatigue, and depression related to its systemic effect. Interleukin-2 (IL-2, Aldesleukin, Proleukin; Novartis) was the second exogenous cytokine to demonstrate anti-tumour activity against melanoma. High-dose bolus intravenous IL-2 was shown to have anti-tumour effects with or without lymphokine-activated killer cells in eight clinical trials conducted between 1985 and 1993, involving 270 patients with advanced metastatic melanoma (Atkins, Lotze et al. 1999). There was an objective response rate of 16% (median response duration, 8.9 months) with a durable response rate of 4%, suggesting that a memory T-cell response was established. This provided a solid basis for the approval by the US FDA in 1998 for the treatment of adults with advanced metastatic melanoma and renal cell carcinoma. The majority of toxicities associated with high-dose IL-2 are severe but reversible, including hemodynamic complications, autoimmunity and thyroid dysfunction. These cytokines were also exploited in co-expressing- (Dummer, Rochlitz et al. 2008), combination- (Yockman, Kim et al. 2007; Rodriguez, Ryu et al. 2008), or adoptive-cell therapy (Heemskerk, Liu et al. 2008).

### 1.1.4.3 Peptide vaccines

Since the discovery of the tumour-derived peptide, the immunogenic motif in cytotoxic T-cell (CTL) stimulation, many studies have used peptides derived from documented tumour-associated antigens, specifically designed to associate with T cells in the context of specific MHC class I or II molecules (Brinkman, Fausch et al. 2004). Several clinical trials in different tumour types have been conducted utilising this vaccination strategy. The majority of these trials indicated that peptide vaccination has few toxicities associated with its administration, but disparities exist between *in-vitro* and clinical responses. A clinical study in patients with melanoma was disappointing despite 91% of patients demonstrating CTL responses after gp100<sub>209-217</sub> peptide vaccination with incomplete Freund's adjuvant (Rosenberg, Yang et al. 1998). Compared with chemotherapy in randomised phase III studies, complex melanoma vaccines, such as Allovectin-7 (Vical Inc, San Diego, CA), Canvaxin, and Melacine (Corixa Corp, Seattle, WA), failed to meet primary end points of improved response or survival (Mitchell 1998). Various strategies in attempts to improve the efficacy of peptide vaccination have been proposed, such as: use of adjuvant cytokines (Jager, Ringhoffer et al. 1996; Rosenberg, Yang et al. 1998), improving immunogenicity by peptide sequence alteration (Oka, Tsuboi et al. 2004), use of tumour antigen or tetanus toxoid sub-epitope fusion DNA sequences (Rice, Elliott et al. 2001), or the incorporation of bacterial proteins as adjuvants (Miconnet, Coste et al. 2001). These strategies have been carried out to augment the immune responses or minimise tumour escape in preclinical and clinical work using peptide vaccination.

Some limitations and uncertainties concerning the mechanism of action and unexplained observations were made when applying TSA-derived peptides as targets. For example, the clinical effects or *in-vivo* immune responses in patients receiving peptide vaccination are not universally proportional to the regression, persistence or upregulation of targeted tumour antigen expression (Ohnmacht, Wang et al. 2001). In addition, the efficacy of the peptides developed for immunotherapy could be restricted by their low immunogenicity or low affinity for cell-surface HLA antigen molecules (Ohnmacht, Wang et al. 2001). Also, peptide vaccination can be used only in the context of appropriate HLA antigen restriction. Peptides can undergo rapid clearance and are dependent on intact antigen-presenting and effector-cell populations to induce a response. Therefore, the clinical effect might be unsatisfactory due to a compromised host immune system. Finally, tumour cells can evade recognition through the downregulation or alteration of a single gene product that is not essential for maintaining the malignant phenotype.

#### **1.1.4.4 Plasmid DNA and recombinant viral vaccines**

Since most types of cancer have a genetic predisposition, and as a result of the identification of various tumour suppressor genes or oncogenes and advances in recombinant gene technology, cancer gene therapy offers a number of exciting potential treatments. Gene therapy encompasses a wide range of treatment types, all of which use genetic material to modify cells (either *in vitro* or *in vivo*) to augment anti-tumour response or to promote apoptosis of tumour cells. Naked DNA, viral or non-viral vectors engineered to express the desired gene are frequently used for this purpose. Vaccination can ultimately result in the incorporation of transgenes into host

antigen-presenting cell (APC) for endogenous processing and presentation. However, the responses from naked DNA vaccination were weak and not reproducible (Vollmer, Eilber et al. 1999). Addition of cytokine genes to the DNA vaccine has led to enhanced tumour protection (Sedegah, Weiss et al. 2000; Fernandez, Zeiser et al. 2007). Recently a diversified prime or boost strategy, boosting by a different vector encoding the same tumour gene, was demonstrated to be more potent than using repetitive vaccinations with the same vector alone. Combining DNA plasmids expressing AFP and GM-CSF with subsequent boost vaccination with a non-replicating adenoviral vector encoding the same tumour antigen has also enhanced antigen-specific tumour protection (Meng, Butterfield et al. 2001).

The field of cancer gene therapy is maturing rapidly and several cancer vaccine treatments have entered late stage clinical trials. In addition, gene transfer technology for cancer treatment holds great promise for increasing the effectiveness of current chemotherapeutic treatment regimens. Significant advances have been made in the field of oncolytic virotherapy, and trials are in progress that incorporate this technique for precancerous, as well as cancerous, treatment (Collison, Workman et al. 2007).

#### **1.1.4.5 Dendritic cell-based vaccines**

Dendritic-cell (DC)-based vaccines are the newest development in cancer vaccine design. DCs can be loaded with autologous or allogeneic tumours, apoptotic bodies, tumour lysates (Morgan, Dudley et al. 2006), tumour RNA or DNA (Nestle, Banchereau et al. 2001). Initial preclinical studies involved pulsing of DC with tumour-specific peptides to induce antigen-specific responses (Mayordomo, Zorina et al. 1995). A phase I study of a vaccine composed of DCs that were loaded with mRNA

encoding prostate-specific antigen (PSA) induced T-cell responses against PSA in most patients and the log slope of PSA was temporarily decreased, indicating perhaps that tumour growth was reduced (Heiser, Coleman et al. 2002). Although evidence for the safety and *in-vivo* bioactivity of RNA-loaded DCs in patients with metastatic prostate cancer was suggested, proof of clinical benefit remains to be established in future clinical trials. A strategy for prolonged presentation of an MHC class I-restricted self-peptide on DCs, through its linkage to a cell penetrating peptide, (CPP) was introduced (Wang and Wang 2002). Another strategy involves the loading of DC with whole tumour proteins (Timmerman, Czerwinski et al. 2002). Gene transfer methods offer an alternative means of introducing tumour antigens into DC using plasmid DNA, RNA, and viral vectors (Nair, Boczkowski et al. 2007). A study from Zhu et al demonstrated that a vector simultaneously over-expressing three costimulatory molecules could be used efficiently to infect human DCs, leading to enhanced peptide-specific T-cell activation (Zhu, Terasawa et al. 2001).

Peptide vaccination depends on the loading of empty MHC molecules on APCs *in vivo*. However, single administration of peptide without a means of targeting activated APCs can potentially lead to loading of MHC class I molecules on nonprofessional APCs, which could result in tolerance (Toes, van der Voort et al. 1998; Diehl, den Boer et al. 1999). In this case, vaccination with antigen-pulsed DCs is superior to peptide-based vaccination protocols.

#### **1.1.4.6 Adoptive T cell therapy**

The discovery that human tumour-infiltrating lymphocytes (TILs) derived from patients with a variety of types of cancer (including metastatic melanoma, breast cancer, colon cancer and ovarian cancer) can exhibit specific tumour lysis or cytokine

release *in vitro* led to a new therapeutic approach. Adoptive transfer of TILs, after *in vitro* expansion in a way that preserved their anti-tumour activity, was shown to treat murine malignancies effectively, leading to early trials of adoptive transfer of TILs plus IL-2 in humans (Rosenberg, Yannelli et al. 1994). In 1994, Rosenberg et al reported a 34% objective response rate among patients with metastatic melanoma who were treated with adoptive cell therapy (Rosenberg, Yannelli et al. 1994). These results have steadily improved. Adding lymphocyte-depleting chemotherapy before adoptive cell therapy increased the objective-response rate to 49% (Dudley, Wunderlich et al. 2002), and adding radiotherapy increased the objective-response rate to 72% (unpublished data by Rosenberg et al (Rosenberg, Dudley et al. 2008)). With the use of cancer vaccines, the objective-response rate ranges from 3 to 7% (Rosenberg, Yang et al. 2004). Apart from the convincing evidence of adoptive CD8+ cell transfer in anti-tumour effect, *ex-vivo* expanded tumour antigen-specific autologous CD4+ cell therapy also lead to durable tumour remission (Hunder, Wallen et al. 2008). Qiao et al recently demonstrated the possibility of combining adoptive T-cell therapy with oncolytic viral delivery. In mouse models, they showed that antigen-nonspecific T cells loaded with oncolytic vesicular stomatitis virus efficiently delivered the virus to metastatic lymph nodes and lead to tumour clearance associated with anti-tumour immune priming (Qiao, Kottke et al. 2008).

#### **1.1.4.7 Monoclonal antibodies and small molecule inhibitors**

With the advances in understanding of aberrant signalling pathways in various types of cancer cells, many pivotal regulators of malignant behaviour in cancer cells have emerged as candidates for molecular target-based cancer therapy. Such strategies have improved the management of cancers and many of them have been approved

by FDA. The two main approaches include monoclonal antibodies (mAbs) and small molecule inhibitors (Imai and Takaoka 2006). Key signalling molecules, such as protein tyrosine kinases, have proven to be good targets with clinically-effective responses in chronic myeloid leukaemia (CML), gastrointestinal stromal tumours (GISTs) and non-small-cell lung cancer (NSCLC). Another group of targets is represented by tumour-selective cell-surface proteins, which can be recognised by mAbs, such as CD20 (Rituximab) (Coiffier 2006), Gemtuzumab (CD33) and HER2/Neu (trastuzumab) (Vogel, Cobleigh et al. 2002). Many of these mAbs have been recommended as standard therapies singly, or combination, to traditional cytotoxic chemotherapy. Interestingly, they are specific receptor inhibitors or B-cell derived mAbs, instead of therapeutic vaccines to activate CTLs, and have passed the acid test of clinical trials and become standard anti-cancer regimens.

Thanks to the advances in unraveling the molecular interactions between tumours and the immune system, many immune-based cancer targeting approaches in enhancing APC/T-cell responses or antagonising regulatory pathways, so-called the immune checkpoint blockade that induce immune tolerance or suppressor circuits, have been developed (Cheever 2008). Anti-CD40 and CD40L are potent agonists for increasing APC function. Preclinical studies have demonstrated that agonist CD40 monoclonal antibody (mAb) can mimic the signal of CD40L, substitute for the function of CD4<sup>+</sup> lymphocytes, overcome T-cell tolerance, and direct tumour inhibition (Vonderheide, Flaherty et al. 2007). Anti-CD137 (anti-4-1BB), which against a member of the TNF superfamily of receptors 4-1BB, has been shown its potent T-cell stimulation and enhance CTLs responses to vaccine by agonistic mAb (Melero, Shuford et al. 1997; Wilcox, Flies et al. 2002). Programmed death-1 (PD-1) is a negative regulator of T-cell function and blocking PD-1 can increase anti-tumour T-



cell immunity and induce long-lasting tumour regression (Hirano, Kaneko et al. 2005). 1-Methyl tryptophan (1MT) is also one of the small molecule inhibitors in overcoming tumour-related immunosuppression by enzyme indoleamine 2,3-dioxygenase (IDO) (Uyttenhove, Pilotte et al. 2003). These agents are being studied in combination with convention chemotherapies or other immunotherapy strategies.

### **1.1.5 Obstacles in cancer vaccine development**

There are many factors that influence tumour response to immunotherapy. Neoplastic cells survive and proliferate in an often hostile environment. Under constant selection pressure, cells with a survival privilege ensure that the tumour “evolves” to suit its environment. The concept of tumour cells developing mechanisms to evade the immune system and becoming immunoresistant is referred to as cancer immunoediting (Dunn, Bruce et al. 2002; Dunn, Old et al. 2004). Within this context, the ability of immune system to attack cancer cells is limited.

Although great progress has been made in the development of cancer vaccines in the past few years, many challenges remain when transferring from experimental results to clinical practice and to curing cancer (Ostrand-Rosenberg 2004). In testing in mice, effective immunity can often be elicited, with a successful inhibition of tumour growth following immunisation. Also, minimal toxicity and evidence of immunologic response have been demonstrated but only anecdotal responses to several different vaccine strategies in clinical trials have been reported. A clear correlation between immunologic and clinical response has not been consistently observed (Borges, Kufe et al. 2002). In Rosenberg et al’s review, an overall tumour response rate was only 2.6% from hundreds of vaccine clinical trials (Rosenberg, Yang et al. 2004). This poor efficacy is due to a large number of parameters or mechanisms that prevent

tumour destruction by immune cells (Rosenberg 2008). These parameters are: inadequate numbers or avidity of the immune effector cells, the inability of the tumour to activate quiescent or precursor lymphocytes, tolerance mechanisms including anergy, and suppressor influences produced by the tumour or the immune system itself (Marincola, Jaffee et al. 2000; Rosenberg 2008).

One of the numerous peptide vaccines focusing on metastatic melanoma developed by Corixa Corp. (Melacine®) could delay the tumour growth in vaccinated patients but was restricted to a particular HLA antigen (MHC-A2 or C3) (Sosman and Sondak 2003). The use of cellular-based therapies (e.g., DC-based vaccines) appears promising, but involves technical challenges and requires sophisticated cell manipulation. Although DC pulsed with peptides appear to be good candidates for a clinical use in human tolerisation (Dallal and Lotze 2000), *ex-vivo* approaches suffer from two problems, one related to the generation of high numbers of clinically exploitable DCs, and the other one resulting from multiple rounds of immunisation at fairly short intervals that can at times lead to the emergence of Th1/Th2 cells imbalance and non-cytolytic CD4+ cells. Gene therapy with viral vectors expressing tumour antigen is potentially limited by the generation of the host-immune response to the vector itself, although a higher level of immune stimulation, as compared with naked DNA, can be achieved. In addition, antiviral response leads to rapid clearance of the vector in subsequent vaccinations and, thereby, prevents augmentation of the immune response.

These obstacles need to be overcome in future vaccine development and, additionally, vaccination schedule, mode of delivery, adjuvants, and cofactor molecules should be optimised. Meanwhile, all the immunologic endpoints should also be carefully documented (Simon, Steinberg et al. 2001).

### 1.1.6 Regulating the immune responses to tumours

A major impediment to cancer immunotherapy is tumour-induced immune suppression and tumour evasion of anti-tumour immune responses, which ultimately render the host tolerant to tumour-associated antigens. Several limiting factors will affect the efficacy of immunotherapeutic strategies such as DC activation, antigen presentation, and cancer cell recognition. In addition, local immunosuppressive factors within the tumour milieu include secreted molecules, such as TGF- $\beta$ , IL-10, prostaglandin E2, and vascular endothelial growth factor (VEGF). These factors are produced either by the tumour cells themselves or by surrounding host stromal cells on tumour cell signalling. The suppressive tumour environment is further maintained by immunosuppressive cells, such as T regulatory cells (Treg), and indolamine 2,3-dioxygenase (IDO)-producing plasmacytoid DC (Munn and Mellor 2004). These are professional immune suppressor cells that are frequently found inside tumours and provide means for tumour escape from activated effectors.

Current strategies for cancer immunotherapy focus mainly on enhancing T-cell mediated tumour lysis; however, a variety of active mechanisms may limit the effectiveness of this approach. The past 10 years have witnessed a renaissance in the field of active mechanisms of immune regulation, and much of this points to a particular group of T cells, currently described as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells which play a suppressive role in anti-tumour vaccinations (Kronenberg and Rudensky 2005). Liyanage et al and others have shown that an increased prevalence of suppressor T cells in the tumour microenvironment of patients with invasive cancers can suppress a tumour-specific immune response (Liyanage, Moore et al. 2002). Many mouse studies have shown that using monoclonal antibodies (PC-61) depleting

CD25<sup>+</sup> cells promotes immune responses induced by tumour cells or antigen-pulsed dendritic cells as well as immune-mediated rejection of tumour cell-lines (Onizuka, Tawara et al. 1999; Prasad, Farrand et al. 2005). More impressively, intra-tumoural injection of CD4-specific antibodies promoted regression of established tumours (Yu, Lee et al. 2005) indicated that tumour infiltrated CD4<sup>+</sup>CD25<sup>+</sup> cells dampened local immune anti-tumour responses. In a separate study using a different tumour cell line, intra-tumour depletion of CD25<sup>+</sup> cells was shown to inhibit tumour growth and the delay of tumour growth was extended by multiple rounds of depletion (Needham, Lee et al. 2006). Another effective approach tested in rodent models, the use of cyclophosphamide to deplete CD4<sup>+</sup>CD25<sup>+</sup> cells in combination with tumour cell vaccination, led to regression of established tumours in rats (Ghiringhelli, Larmonier et al. 2004). Blocking antibodies to CTLA-4 are in clinical development and have shown durable immune-mediated response rates in the order 5% to 20% in patients with metastatic melanoma (Downey, Klapper et al. 2007; Ribas, Hanson et al. 2007).

The increasing number of studies of Treg cells in patients with cancer also point to a role for these cells in promoting cancer progression (Betts, Clarke et al. 2006). There is, therefore, a clear rationale for developing clinical strategies to manipulate these regulatory influences, with the ultimate goal of augmenting anti-tumour immunity (Lizee, Radvanyi et al. 2006).

### **1.1.7 Potential of combined immunotherapy and chemotherapy**

The integration of immunotherapies with conventional treatments for cancer is a challenge, especially for cancer vaccines, because of the immunosuppressive effects of most standard treatments. However, the exploration of combined treatments is revealing unexpected results. The synergistic activity of the combinations is

supported by recent clinical and pre-clinical studies (Chong and Morse 2005). Although the combination would at first seem to be counterproductive, supportive data have revealed that cyclophosphamide, gemcitabine and doxorubicin enhance the efficacy of vaccines when used as a form of 'pretreatment'. In a review of three prostate cancer vaccine trials, researchers from the National Cancer Institute (NCI) offered evidence that prior immunotherapies can sensitise the tumour to subsequent chemotherapy (Wheeler, Das et al. 2004; Schlom, Arlen et al. 2007). Another study demonstrated an apparent survival advantage for patients treated with the vaccine followed by chemotherapy as compared to chemotherapy alone (Antonia, Mirza et al. 2006; Inoges, Rodriguez-Calvillo et al. 2006). All of these reports support the concept that a properly "conditioned" host's immune system might be achieved by the combination.

The mechanisms responsible for this observed improvement in immunotherapy with chemotherapy remain mostly unknown and need to be explored. The elimination of cells with immunosuppressive activity, especially Treg, non-specific activation of APC, improved cross-presentation of tumour antigens, lymphodepletion, and resultant homeostatic T cell proliferation have been proposed. In addition, other local effects have been speculated: disruption of tumour stroma that results in improved penetration of CTLs into the tumour site, decreased local suppressive activity of tumour cells, increased permeability of tumour cells to CTL-derived granzymes, increased expression of tumour associated antigens by tumour cells, and up-regulation of death receptors (such as Fas) on tumour cells (or FasL on CTLs) (Ramakrishnan, Antonia et al. 2008). This combination may provide substantial clinical benefits for patients with advanced disease, since active or adoptive immunotherapies seem to be incapable of controlling large tumour masses. Further

clinical trials will attempt to optimise combinations through the selection of treatment modalities, the dosage of drugs, and the time-scheme of combination therapies.

### **1.1.8 Perspective**

One of the hallmarks of cancer responses to cellular immunotherapy is that they are extremely long lived, frequently measured in years. However, objective responses (as opposed to the more common claim of mixed responses or disease stabilisation with immunotherapy) are rare, usually noted in a minority of patients, in the range of 5% to 10% (Rosenberg, Yang et al. 2004). In a review of randomised phase III studies with active cancer immunotherapies, most have failed to show a significant benefit with respect to predetermined end points (Finke, Wentworth et al. 2007). New strategies are being explored (Finn 2003; Rosenberg, Yang et al. 2004), including vaccines based on engineered viral vectors, various approaches with dendritic cells, and strategies that are aimed at inhibiting immunosuppressive cells of lymphoid or myeloid origin.

Based on previous experiences in developing cancer vaccines, future approaches of cancer immunotherapy should focus on the development of the following strategies:

- Choose the right target: to stimulate specific CTLs, increase the number of antigen-expressing DCs, enhance MHC class I presentation of the encoded antigen.
- Use appropriate adjuvant/vehicle to safely, easily and powerfully boost the frequency and magnitude of immune responses in immuno-compromised patients.
- Immune responses, tailored to aging immune system and inhibition of Treg cells function-allowing to overcome tumour-induced, therapy-induced, or age-induced immunosuppression.

- Optimal immunisation schedule to induce appropriate antigen presentation in a short priming phase (den Boer, Diehl et al. 2001), avoid overstimulation which results in Treg cells activation (Darji, Guzman et al. 1997) and elicitation of long-term memory.
- Combination approaches to yield synergistic or additive results (Cuadros, Dominguez et al. 2003; Mihich 2003; Morgan, Dudley et al. 2006; Offringa 2006).
- Strategy to modulate apoptotic pathways in cancer cells, such TRAIL receptor or Fas, to synergise with immunotherapy and other targeted therapy approaches. A soluble TRAIL ligand construct and TRAIL receptor-activating antibodies are in clinical development for the treatment of cancer. These approaches may be viewed as bypassing the need for immune effector cells.

## **1.2 BACTERIA AND GENE THERAPY**

### **1.2.1 Bacteria and cancer**

Certain bacteria are associated with human cancers, for example, *H. pylori* and gastric cancer or MALT lymphoma, *Salmonella typhi* and gallbladder cancer (Lazcano-Ponce, Miquel et al. 2001), *Chlamydomphila pneumoniae* and lung cancer (Littman, Thornquist et al. 2004), *Streptococcus bovis* and colorectal cancer (Biarç, Nguyen et al. 2004). Their roles in carcinogenesis, however, are still unclear. Convincing evidence links some species to carcinogenesis while others appear promising in the diagnosis, prevention or treatment of cancers. Research has shown that exposure to *H. pylori* appears to reduce the risk of oesophageal cancer; however *S. typhi* is a promising carrier of therapeutic agents for melanoma, colon and bladder cancers (Niethammer, Xiang et al. 2001; Mager 2006). Spontaneous tumour

regression has followed severe bacterial, fungal, viral and protozoal infections and thereby inspired the development of the earliest cancer therapies (Richardson, Ramirez et al. 1999).

### **1.2.2 Use of bacteria as gene-delivery vectors**

In the past, direct injection of naked DNA has been shown to allow transgene expression in muscle (Wolff, Malone et al. 1990). However, for most gene therapy applications, the need for an efficient and relevant vector for gene transfer is now widely acknowledged. Classically, these vectors are classified into non-viral and viral vectors. The non-viral vectors are usually chemically defined compounds such as liposomes that complex the genetic material and mediate gene transfer (Glover, Lipps et al. 2005). Viral vectors, replicating or not, are recombinant viruses in which all or part of the viral genome has been replaced by the expression cassette encoding the therapeutic transgene. Retroviruses, adenoviruses, poxviruses, parvoviruses and herpesviruses belong to the most frequently used viral factors (Celec, Gardlik et al. 2005). Gene transfer can also occur from bacteria to a very broad range of recipients that include yeast (Heinemann and Sprague 1989) and plants (Lessl and Lanka 1994) and several laboratories have reported a relatively high frequency of functional gene transfer from bacteria to mammalian cells (Sizemore, Branstrom et al. 1995; Darji, Guzman et al. 1997; Dietrich, Bubert et al. 1998; Grillot-Courvalin, Goussard et al. 1998; Paglia, Medina et al. 1998; Loessner and Weiss 2004).

Bactofection, using bacteria for direct gene transfer into the target organism, organ or tissue, is a novel approach which exploits intracellular bacteria as delivery vectors for plasmid DNA. Distinct advantages of such carrier-based DNA vaccines



are that: (1) these carriers specifically target APCs at the inductive sites (i.e. the most effective target) (2) live attenuated vaccines induce the secretion of several cytokines and proinflammatory mediators that enhance early innate immunity and create a local environment conducive to antigen presentation and (3) bacterial cell components and unmethylated DNA as so-called pathogen-associated molecular patterns (PAMPs) provide a strong “danger signal” through the activation of pattern recognition receptors on APCs.

Stimulation of APC with PAMPs will trigger an innate immune response in the form of the production of reactive oxygen, pro-inflammatory cytokines, and nitrogen species, as well as up-regulation of co-stimulatory molecules. These responses promote the maturation and migration of DCs to secondary lymph nodes (Banchereau and Steinman 1998) and, in this way, PAMPs amplify the immune response against the antigen and act as adjuvants. PAMPs exert their action through binding to receptors of the Toll-like receptor (TLR) family (Banchereau and Steinman 1998). PAMPs include compounds such as lipopolysaccharide (LPS) (Poltorak, He et al. 1998; Poltorak, Ricciardi-Castagnoli et al. 2000), bacterial DNA-containing unmethylated cytosine-phosphate-guanine (CpG) dinucleotides (Hemmi, Takeuchi et al. 2000; Stetson and Medzhitov 2006), flagellin (Gewirtz, Navas et al. 2001) or bacterial lipoproteins (Aliprantis, Yang et al. 1999).

### **1.2.3 Mechanisms of “cargo” delivery by bacterial vectors**

The first step of gene transfer by bacterial vectors lies in the entry of the delivery vehicle uptake by host target cells. The bacterial cell is typically confined to spherical or cylindrical shapes between 1 and 5  $\mu\text{m}$  which can innately target the phagocytic cells. When professional phagocytic cells such as macrophages or dendritic cells are

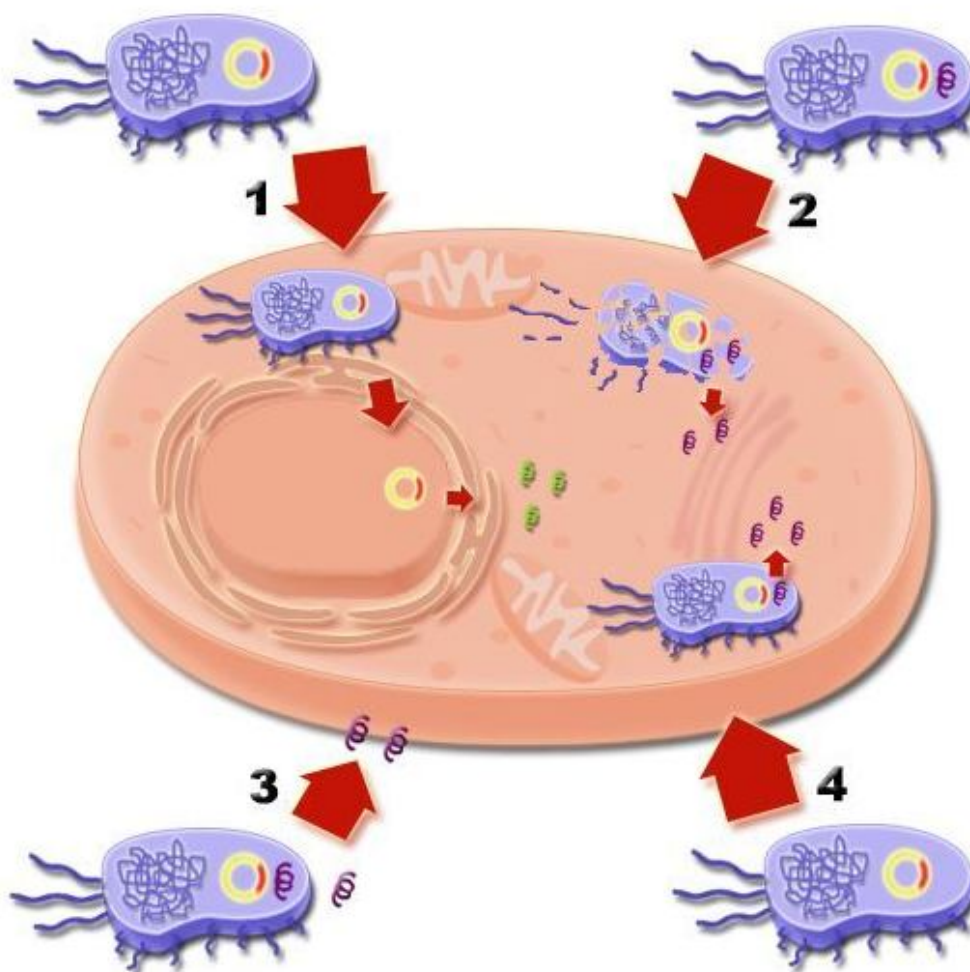
targeted, this entry is likely to happen through phagocytosis. This contact will activate these phagocytic cells, i.e. the APCs. Gram negative cells contain LPS, whereas Gram-positive cells possess lipoteichoic acid. These and other PAMPs naturally influence bacterial uptake and act as natural adjuvants to improve APCs activation through binding to TLR (Hemmi, Takeuchi et al. 2000; Poltorak, Ricciardi-Castagnoli et al. 2000). In the case of non-phagocytic cells, such as those of the intestinal epithelium, there are two major strategies for bacteria to gain entry into a eukaryotic cell (Marra and Isberg 1996; Poltorak, He et al. 1998). For certain genera such as *Salmonella* or *Shigella*, contact between the bacteria and the host results in the secretion by the bacteria of a set of invasion proteins that triggers intracellular signalling events. For example, contact of *S. typhimurium* with host cells results in activation of a specialised protein secretion system (type III) which leads to cytoskeletal rearrangement, membrane ruffling, and bacterial uptake by pinocytosis (Poltorak, He et al. 1998). For other genera such as *Yersinia* or *Listeria*, binding of a single bacterial protein to a particular ligand on the host cell surface is necessary and sufficient to trigger entry by a zipper-like mechanism. After internalisation, the bacteria are localised in the phagosomal vacuoles and are targeted for degradation. Therefore, escaping from the vacuolar compartment is essential to the delivery of DNA vaccines (Figure 2 (1)).




*Shigella spp.* and *L. monocytogenes* can disrupt the endosomal/lysosomal membrane, escape into the host cell's cytoplasm and spread from one infected cell to an adjacent one by exploiting the cell's actin polymerisation for intracellular motility (Dietrich, Bubert et al. 1998; Ogawa and Sasakawa 2006). This process of phagosomal escape is particularly well understood and exploited in vectors based on bacteria replicating in the cytosol (Dietrich, Bubert et al. 1998; Grillot-Courvalin,

Goussard et al. 2002; Birmingham, Canadien et al. 2008). In *L. monocytogenes*, the pore-forming cytolysin listeriolysin-O (LLO), which is encoded by *hly*, plays an essential role in the escaping step. Strains with mutations in *hly* that inactivate the pore-forming activity of LLO are unable to escape from the primary phagosome (Gaillard, Berche et al. 1987; Michel, Reich et al. 1990). Once spread into the cytosol, wild-type *L. monocytogenes* will replicate (Portnoy, Chakraborty et al. 1992). An attenuated *L. monocytogenes* strain has been engineered to undergo self-destruction in the cell cytosol by production of a phage lysine under the control of the promoter of *actA*, which is preferentially activated when the bacteria are in the cytosol (Dietrich, Bubert et al. 1998; Brockstedt, Bahjat et al. 2005). In the case of bacterial delivery vectors that remain in the lysosome/phagosome, such as *Salmonella*, the mechanism of delivery remains unclear (Grillot-Courvalin, Goussard et al. 1999).

Another interesting approach might be used in bacterial delivery systems (Palffy, Gardlik et al. 2006). Bacteria are not used for the gene transfer but the proteins secreted by bacteria persist in the target tissues (Figure 2 (2)). More recently, the utilisation of double-stranded RNA (dsRNA or RNA interference, so-called RNAi) to silence target genes has potential therapeutic applications that are widely acknowledged (Karagiannis and El-Osta 2005; Vassaux, Nitcheu et al. 2006). Recent progress in this strategy was made using nonpathogenic bacteria to induce gene silencing in target cells, both *in vitro* and *in vivo*. Bacteria-mediated induction of RNAi was established by demonstrating target-specific gene silencing after transfer of double-stranded RNA from *E. coli* in the nematode *Caenorhabditis elegans* (Timmons and Fire 1998; Timmons, Court et al. 2001). Another example of bacteria-mediated RNAi transfer for functional genomics is postulated by Xiang et al who developed an siRNA delivering system using nonpathogenic *E. coli* engineered to

transcribe shRNAs from a plasmid containing the invasin gene *inv* and the LLO gene *hly*, which encode two bacterial factors needed for successful transfer of the shRNAs into mammalian cells. Upon oral or intravenous administration, *E. coli* encoding shRNA against catenin  $\beta$ -1 (CTNNB1) induce significant gene silencing in the intestinal epithelium and in human colon cancer xenografts in mice (Li, Parker et al. 2006; Xiang, Fruehauf et al. 2006). In another *in-vivo* study carried out by Zhang et al, using attenuated *S. typhimurium* to deliver STAT3-specific RNAi, bacterial vector could preferentially home to tumour tissues, inhibit tumour growth, and extend survival time in experimental mice (May, Dao et al. 2007). In the light of these results obtained previously, the delivery of dsRNA or eukaryotic expression plasmids encoding siRNAs into mammalian cells can be envisaged in the near future.



**Figure 2. Different strategies are undertaken by the bacterial vectors in delivering the DNA vaccines.** (1) After bacterial vectors penetrate into the mammalian cells, the vectors are destroyed or undergo lysis in the cytoplasm of target cells. The plasmid carrying the therapeutic gene (  ) is released, enters the nucleus and is expressed by the eukaryotic transcription and translation machinery (  ). (2) The passenger antigen (  ) is expressed within the bacterial compartment and delivered into the target cells. These non-secretory antigens can only be released into the cytoplasm of the target cell upon phagosomal lysis of the vector. Further antigen processing and presentation will happen essentially in antigen-presenting cells (APC). (3) Bacterial vectors do not enter the eukaryotic cell, but express and secrete the therapeutical transgene in the intercellular space. (4) The bacterial vector can escape from lysosomal/phagosomal lysis. The transgene is expressed by the prokaryotic transcription and translation machinery after penetrating into the cytoplasm of the target cell, and the expressed antigenic protein is secreted into the cytoplasm.

#### 1.2.4 Protein vs. Gene therapy

Another approach that might be used in gene therapy is the so-called alternative gene therapy (AGT) (Celec, Gardlik et al. 2005; Palffy, Gardlik et al. 2006). Bacteria are not used for the gene transfer but persist in the target tissues. Persisting bacteria maintain the therapeutic polypeptide *in situ*, thus, this technique resembles bacterial protein delivery (Figure 2 (4)). In comparison to classic gene therapy using gene transfer into the mammalian cells, AGT offers the possibility of regulation of gene expression using low molecular weight inducers of expression dependent on the expression system used. Celec et al demonstrated that the transformed *E. coli* expressing VEGF (in the T7 expression system) could induce blood vessel formation in mice after intraperitoneal injection (Celec, Gardlik et al. 2005). If needed, the therapy can always be stopped. Bacteria can be eliminated using antibiotics as their resistance spectrum is defined. This negative regulation cannot be performed in

classic gene therapy or bactofection. The use of antibiotics in bactofection or suicide genes (e.g. thymidine kinase) in viral vectors can increase the safety of delivery, but does not affect the expression of therapeutic genes. AGT can be improved using experience from bactofection experiments since the transport of bacteria in the organism does not differ significantly between these two methods. Although bactofection and AGT share some characteristics such as their side effects and other similarities, the key difference lies in the expression of the desired gene. In bactofection, the transgene is expressed in the eukaryotic host cell; in AGT bacteria are the producer of the therapeutic peptide.

### 1.2.5 Application of bacterial vectors in cancer immunotherapy

Anecdotal case reports from more than 200 years ago describe tumour regression in patients with severe bacterial infections (Hall 1998). Application of bacteria in cancer therapy was pioneered independently by Friedrich Fehleisen and William B Coley in the late 1800s and early 1900s (Coley 1893), leading eventually to immunomodulation for the treatment of cancer. Many more recent studies have shown the potential of genetically engineered bacteria as tumour-targeting vectors in human cancer therapy (Table 1). Among the bacterial vectors designed for cancer vaccines, *Salmonella* strains have essentially been used to deliver DNA for therapeutic applications in oncology. In the first instance, 'model' tumour antigens such as  $\beta$ -galactosidase were encoded in eukaryotic expression vectors carried by strains of *Salmonella* (Paglia, Medina et al. 1998; Weth, Christ et al. 2001). Oral administration of these bacterial strains to deliver DNA vaccines encoding the model tumour antigens protected the mice against challenges with fibrosarcoma (Paglia, Medina et al. 1998) or renal carcinoma (Weth, Christ et al. 2001). In addition, when real murine/human tumour antigens/epitopes were expressed in *Salmonella* vector, the tumour protective effect could still be demonstrated in neuroblastoma (Xiang, Lode et al. 2000; Huebener, Lange et al. 2003; Pertl, Wodrich et al. 2003), melanoma (Weth, Christ et al. 2001; Cochlovius, Stassar et al. 2002) and adenocarcinoma (Niethammer, Primus et al. 2001; Zhou, Luo et al. 2004).

Attacking the tumour's vasculature has been documented as an effective strategy to inhibit tumour growth/metastasis. This antiangiogenic intervention has been demonstrated in *Salmonella* expressing vascular endothelial growth factor receptor (FLK-1 or Fra-1) (Niethammer, Xiang et al. 2002; Reisfeld, Niethammer et al. 2004; Reisfeld, Niethammer et al. 2004; Zhou, Luo et al. 2005; Luo, Markowitz et al.

2007). Recently, Endoglin (CD105), a co-receptor in the TGF- $\beta$  receptor complex that is overexpressed on proliferating endothelial cells in the breast tumour neovasculature, was delivered by attenuated *S. typhimurium*. In a prophylactic setting, a pronounced CD8<sup>+</sup> T cell response was induced which effectively suppressed dissemination of pulmonary metastases in mice (Lee, Mizutani et al. 2006; Needham, Lee et al. 2006). Another strategy used the delivery of the endogenous angiogenic inhibitor, thrombospondin-1 (TSP-1), by *S. choleraesuis* was shown to be effective for the treatment of primary and metastatic melanomas (Yu, Lee et al. 2005). Platelet derived growth factor receptor beta (PDGFR $\beta$ ) expressed by a *Salmonella* vaccine also showed the suppression of angiogenesis *in vivo* and reduction of tumour stromal cell proliferation (Kaplan, Kruger et al. 2006). Combined application of cyclophosphamide with a DNA vaccine targeting PDGFR $\beta$  not only completely inhibited the growth of different tumour types but also led to tumour rejections in mice (Loeffler, Le'Negrate et al. 2008). Legumain is highly upregulated on macrophages in many tumour tissues and consequently a valid tumour antigen for immunotherapy. Immunisation of mice with *Salmonella* vaccine encoding Legumain induced a robust CD8<sup>+</sup> T cell response against tumour associated macrophages and in turn led to a suppression of tumour angiogenesis, tumour growth and metastasis by profoundly altering the tumour microenvironment (Luo, Zhou et al. 2006). Emergence of acquired multidrug resistance (MDR) remains a major challenge in the treatment of cancer following chemotherapeutic drugs and, thus, the MDR gene serves as an alternative cancer vaccine target. *Salmonella* vaccines against MDR-1 have also inhibited tumour growth and metastasis in preclinical studies (Niethammer, Wodrich et al. 2005). This also enabled the further



combination of targeting MDR-1 with traditional cytotoxic therapy in effective treatments of cancer.

It has been shown that the immune responses mediated from bacteria delivering DNA vaccines can be dramatically improved by modifying the expression vectors or co-expression of immunostimulatory molecules. Optimisation of the bacterial vectors carrying DNA vaccine was shown to be achieved by several strategies:

- The level of expression of the antigen can be enhanced by adding a post-transcriptional regulatory acting RNA element. In experimental murine models, the anti-tumour effect can be further augmented by boosting with antibody-cytokine fusion protein that targets IL-2 to the tumour microenvironment (Pertl, Wodrich et al. 2003).
- Antigen-fusion to ubiquitin can also increase antigen processing and effective presentation in order to break peripheral T cell tolerance to a self-antigen (Xiang, Lode et al. 2000; Xiang, Primus et al. 2001).
- Co-expression of invariant chain leads to preferential presentation in the context of MHC class II molecules (Weth, Christ et al. 2001).
- Expression of CD40L (Urashima, Suzuki et al. 2000) alone or co-expression of CD40L/antigen (Xiang, Primus et al. 2001) up-regulated the expression of Fas, B7-1, and B7-2 molecules and improved antigen presentation to T cells.
- Fusion of cytokine genes into DNA vaccine produced immune-modulatory effects, such as IL-2 (Niethammer, Xiang et al. 2001), IL-4/IL-18 (Rosenkranz, Chiara et al. 2003; Agorio, Schreiber et al. 2007), GM-CSF/IL-12 (Yuhua, Kunyuan et al. 2001), IL-12/VEGFR2 (or FLK-1) (Feng, Zhao et al. 2005), IFN- $\gamma$  (Paglia, Terrazzini et al. 2000), IL-18 (Luo, Zhou et al. 2003) or combined FRA-1 (Luo, Zhou et al. 2003; Luo, Zhou et al. 2005), CCL21 with apoptosis

protein inhibitor (survivin) (Luo, Zhou et al. 2005), NKG2D ligands (Zhou, Luo et al. 2005; Zhou, Luo et al. 2006), and Fas Ligand (FasL) (Loeffler, Le'Negrate et al. 2008).

- Co-expression of LLO in addition to tumour antigen caused lysosomal escape and the antigenic epitope to be presented onto MHC class I complex (Radford, Higgins et al. 2002; Radford, Jackson et al. 2003; Critchley-Thorne, Stagg et al. 2006).
- From the experience using bacterial vaccine against HIV infection (Gao, Xue et al. 2003; Tsunetsugu-Yokota, Ishige et al. 2007), the use of a prime-boost strategy to enhance and prolong CTL responses is also applicable in the field of cancer vaccines, such as priming with naked DNA vaccine and boosting by bacterial vaccine, or priming with bacterial vaccine boosting by antigen-loaded DCs (Weth, Christ et al. 2001).

### **1.2.6 An alternative approach: Targeting hypoxic tumours using anaerobic bacteria**

Hypoxic or necrotic regions are characteristic of solid tumours in many murine and human tumours, including the majority of primary tumours of the breast and uterine cervix. The limitation of cancer gene therapy approaches in solid tumour treatment is the lack of specific, high-level of expression within tumour tissues or the tumour environment following systemic or parenteral administration. Accordingly, gene therapy for solid tumours that exploits and targets gene expression in hypoxic tumour cells is currently being investigated (Dachs, Patterson et al. 1997). It is known that certain species of anaerobic bacteria, including the genera *Clostridium* and *Bifidobacterium*, can selectively germinate and grow in the hypoxic regions of solid

tumours after intravenous injection (Yazawa, Fujimori et al. 2000; Fujimori 2006). In such cases, bacteria proliferate between tumour cells and thus the therapeutic gene is not introduced into the tumour cell, but rather the therapeutic protein is produced in the tumour (Figure 2 (3)). Candidate bacterial vectors for gene introduction include species of *Bifidobacterium*, *Clostridium* and *Salmonella*. *Bifidobacterium* is a normal bacterial flora in the intestine. The nonpathogenic *B. longum* transformed with plasmid containing the gene for human endostatin, a potent inhibitor of angiogenesis, has been shown to inhibit liver tumour growth in BALB/c mice (Fu, Li et al. 2005). Theys J et al described *C. acetobutylicum* expressing murine TNF- $\alpha$  as a possible tool for cancer therapy (Theys, Nuyts et al. 1999).

The use of anaerobic bacterial vaccine for the selective delivery of pro-drug-activating enzymes has also been proven to be efficient in preclinical studies (Fox, Lemmon et al. 1996; Lemmon, van Zijl et al. 1997; Liu, Minton et al. 2002; Minton 2003). In these studies, the *E. coli* enzyme cytosine deaminase (CD) (Fox, Lemmon et al. 1996; Theys, Nuyts et al. 1999; Liu, Minton et al. 2002) and nitroreductase (Lemmon, van Zijl et al. 1997) were expressed in *Clostridium* and were shown to convert the non-toxic pro-drugs 5-fluorocytosine and CB1954, respectively, into toxic compounds capable of diffusing in the tumours and killing the cancer cells through a bystander effect. Gram-negative *Salmonella* have also been proposed as oncolytic agents. In contrast to obligate anaerobic bacteria such as *Clostridia* and *Bifidobacteria*, *Salmonella* are facultative anaerobic bacteria and have the potential to colonise oxygenated small metastatic lesions as well as large tumours with a hypoxic centre. The anaerobic bacteria *per se* also could exert an oncolytic effect and thus potentiate cytotoxic chemotherapy. In the study by Lee et al, *Salmonella choleraesuis* in combination with cisplatin acted additively to retard tumour growth

and extensively prolong the survival time of mice bearing hepatomas or lung tumours. This enhanced antitumour immune responses, manifested by increased infiltrating neutrophils, CD8+ T cells, and apoptotic cells in the tumours, represents a promising strategy for the treatment of primary and metastatic tumours (Yu, Lee et al. 2005). Dang et al assessed 26 different strains of anaerobic bacteria systemically for their proliferative capacity to grow within avascular compartments of transplanted tumours. One (*Clostridium novyi*) appeared to be particularly promising (Dang, Bettegowda et al. 2001). This research group also demonstrated that intravenous injection of *C. novyi-NT* can potentiate the treatment effect of selected anti-microtubule agents (Dang, Bettegowda et al. 2004) or radiotherapy (Bettegowda, Dang et al. 2003) in animal models, without excessive toxicity. Another study carried out by Cheong et al demonstrated that *C. novyi-NT* plus a single dose of liposomal doxorubicin enhanced the tumour eradication effect (Cheong, Huang et al. 2006).

One study applied this bacterial delivery of prodrug-activating enzyme into a clinical setting (Nemunaitis, Cunningham et al. 2003). Nemunaitis et al used *Salmonella* vector expressing *E. coli* CD in the therapy of chemotherapy-refractory colorectal cancer patients. Although the study was a pilot study with only three participating patients, the results were very promising and point towards the potential of this procedure (Nemunaitis, Cunningham et al. 2003). An earlier study published by Toso et al applying *Salmonella* vaccine (VNP20009) in clinical cancer patients with metastatic melanoma demonstrated that *Salmonella* vaccine could be safely administered intravenously to humans. The maximum tolerated dose was found to be  $3 \times 10^8$  cfu/m<sup>2</sup> with the dose-limiting toxicities being thrombocytopenia, anaemia, persistent bacteraemia, hyperbilirubinemia, diarrhoea, vomiting, nausea, elevated alkaline phosphatase, and hypophosphatemia. Some tumour colonisation was

observed upon injection of the highest tolerated dose. However, no clinical anti-tumour effects were seen in their study (Toso, Gill et al. 2002). Altering the kinetics of infusion failed to improve the response or colonisation in a small number of subsequently treated patients (Heimann and Rosenberg 2003). Another phase I trial of systemic administration of *Salmonella* vaccine to dogs with a spontaneous tumour model showed acceptable toxicity results in detectable bacterial colonisation of tumour tissue and significant antitumour activity in tumour-bearing dogs (Thamm, Kurzman et al. 2005).

**Table 1. Bacterial vectors in experimental cancer treatment**

Bacterial Vector	Encoding antigen or protein	Host immune responses			References
		Humoral	Cellular	Clinical	
<i>Listeria monocytogenes</i>	Influenza virus nucleoprotein	NA	+	+	(Pan, Ikonomidis et al. 1995)
<i>Listeria monocytogenes</i>	Influenza virus nucleoprotein	+	+	+	(Pan, Weiskirch et al. 1999)
<i>Listeria monocytogenes</i>	HPV-16 E7 protein	NA	+	+	(Gunn, Zubair et al. 2001)
<i>Salmonella typhimurium</i>	$\beta$ -galactosidase (model Ag)	+	+	+	(Paglia, Medina et al. 1998; Weth, Christ et al. 2001)
<i>Salmonella typhimurium</i>	gp100 (melanoma)	+	+	+	(Cochlovius, Stassar et al. 2002)
<i>Salmonella typhimurium</i>	gp100 (melanoma)	+	+	-	(Weth, Christ et al. 2001)
<i>Salmonella typhimurium</i>	CD40L	NA	NA	+	(Urashima, Suzuki et al. 2000)
<i>Salmonella typhimurium</i>	Ubiquitinated gp100 and TRP-2 (melanoma)	NA	+	+	(Xiang, Lode et al. 2000)
<i>Salmonella typhimurium</i>	tyrosine hydroxylase (neuroblastoma)	NA	NA	+	(Xiang, Lode et al. 2000; Pertl, Wodrich et al. 2003)
<i>Clostridium sporogenes</i>	Cytosine deaminase	NA	NA	+	(Liu, Minton et al. 2002)
<i>Salmonella typhimurium</i>	Cytosine deaminase	NA	NA	+	(Nemunaitis, Cunningham et al. 2003)
<i>Salmonella typhimurium</i>	murine multidrug resistance-1 (MDR-1)	NA	+	+	(Niethammer, Wodrich et al. 2005)
<i>Listeria monocytogenes</i>	HER-2/neu	NA	+	+	(Singh, Dominiacki et al. 2005)

Bacterial Vector	Encoding antigen or protein	Host immune responses			References
		Humoral	Cellular	Clinical	
<i>Salmonella typhimurium</i>	Survivin and CCL21	NA	+	+	(Luo, Zhou et al. 2005)
<i>Salmonella typhimurium</i>	CEA	NA	+	+	(Niethammer, Primus et al. 2001; Zhou, Luo et al. 2004)
<i>Salmonella typhimurium</i>	Fos-related Ag 1 coexpressing IL-18	NA	+	+	(Luo, Zhou et al. 2003; Luo, Zhou et al. 2005)
<i>Salmonella choleraesuis</i>	Thrombospondin-1 (TSP-1) gene	NA	NA	+	(Yu, Lee et al. 2005)
<i>Salmonella typhimurium</i>	Endoglin (CD105)	NA	+	+	(Lee, Mizutani et al. 2006)
<i>Salmonella typhimurium</i>	NKG2D ligand-pH60 or surviving	NA	+	+	(Zhou, Luo et al. 2005)
<i>Bifidobacterium longum</i>	Endostatin gene	NA	+	+	(Fu, Li et al. 2005)
<i>Salmonella typhimurium</i>	Legumain (Tumour-associated macrophages)	NA	+	+	(Luo, Zhou et al. 2006)
<i>Salmonella typhimurium</i>	Murine PDGF receptor-beta	NA	+	+	(Kaplan, Kruger et al. 2006)
<i>Salmonella typhimurium</i>	Murine vascular endothelial growth factor receptor-2 (FLK-1)	NA	+	+	(Niethammer, Xiang et al. 2002; Luo, Markowitz et al. 2007)
<i>E. coli</i>	Ovalbumin (coexpressing LLO)	+	+	+	(Radford, Higgins et al. 2002; Radford, Jackson et al. 2003; Critchley-Thorne, Stagg et al. 2006)
<i>Salmonella typhimurium</i>	Fas Ligand	NA	+	+	(Loeffler, Le'Negrate et al. 2008)

NA: not available

### 1.2.7 Emerging Technologies for Bacterial Vectors

One of the problems encountered when trying to transfect mammalian cells with large DNA molecules is the possibility of mechanical breakage of these large molecules during the purification process. In this context, the utilisation of bacteria to transfer large DNA molecules would simplify the procedure. The delivery of bacterial artificial chromosomes was first demonstrated into HeLa cells using an invasive *E. coli* (Narayanan and Warburton 2003). Direct DNA transfer of up to around 1 Mb was demonstrated and, as the bacterial vector is equipped with an inducible recombination system, modifications of the bacterial artificial chromosome sequences should be possible. More recently, an alpha-satellite DNA cloned into a P1-based artificial chromosome was stably delivered into the HT1080 cell line and efficiently generated human artificial chromosomes *de novo* (Klink, Schindelbauer et al. 2004). In the same report, a 160 kb construct containing the cystic fibrosis transmembrane conductance regulator (CFTR) gene was transferred into the same cells, where it was transcribed and correctly spliced (Klink, Schindelbauer et al. 2004). In a study in mice, large DNA molecules carrying the viral genome of the murine cytomegalovirus (MCMV) were transferred using *E. coli* and *S. typhimurium* as delivery vectors (Cicin-Sain, Brune et al. 2003). This transfer led to a productive virus infection that resulted in elevated titres of specific anti-MCMV antibodies, protection against lethal MCMV challenge, and strong expression of additional genes introduced into the viral genome. Thus, the reconstitution of infectious virus from live attenuated bacteria presents a novel concept for multivalent virus vaccines launched from bacterial vectors.



The second issue in bacterial vector therapy is to monitor delivery efficiency after vaccination. Soghomonyan et al demonstrated the possibility of using *Salmonella* (VNP20009) expressing HSV-thymidine kinase reporter gene to visualise the anatomical localisation of the bacteria within murine tumours by Positron Emission Tomography (PET). The ability to noninvasively detect *Salmonella* vectors by PET imaging has the potential to be conducted in a clinical setting, and could aid in the development of these vectors by demonstrating the efficiency and duration of targeting as well as indicating the locations of tumours (Soghomonyan, Doubrovin et al. 2005).

The immune system can normally respond to bacterial vector and passenger antigen stimulation but mechanisms of immune regulation in hosts will be initiated to induce self-tolerance. Much of this immune regulation points to a particular group of T cells, currently described as Treg cells, which play a suppressive role in antitumour vaccinations (Kronenberg and Rudensky 2005). Few studies have demonstrated the effects of bacterial adjuvants on Treg cells. Hussain et al demonstrated that Treg cell inhibition is responsible for the differences in vaccine-mediated antitumour responses and less Treg cells were found in mice vaccinated with *Listeria* vector expressing LLO and antigen (Hussain and Paterson 2004; Hussain and Paterson 2005). By the increasing number of studies regarding the role of Treg cells in cancer patients (Betts, Clarke et al. 2006; Lizee, Radvanyi et al. 2006), there is a clear rationale for developing bacterial vaccines to manipulate these regulatory influences to augment antitumour immunity.

## 1.3 LLO-BASED PROTEIN EXPRESSING SYSTEMS IN BACTERIAL GENE THERAPY

### 1.3.1 Using *Listeria monocytogenes* as gene therapy vectors

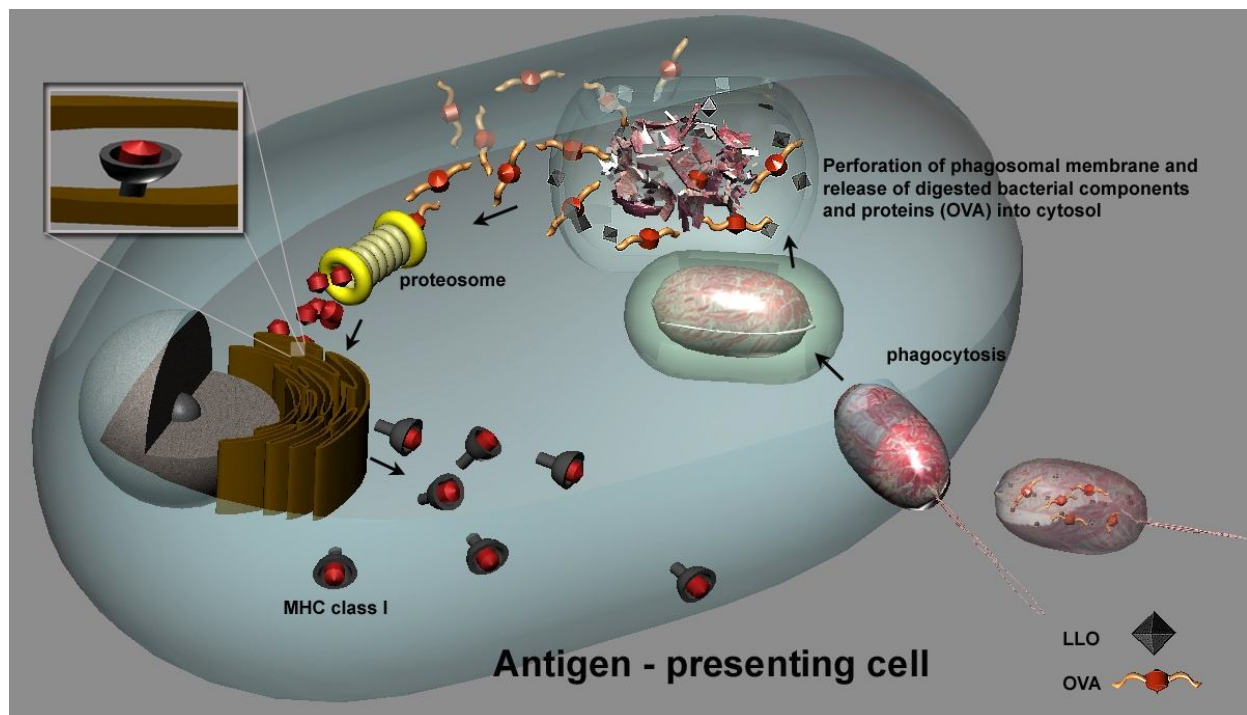
During the past decade, the search for an effective system for the selective delivery of high therapeutic doses of anti-cancer agents to tumours has explored a variety of ingenious and increasingly complex biological systems. Intracellular bacteria are able to generate a strong, cell-mediated immune response within the host. Previous studies have demonstrated the ability of *Listeria monocytogenes* to be used as vaccine vectors in inducing directed immune responses to added antigens (Hussain and Paterson 2005). *L. monocytogenes* is especially attractive, as compared to other intracellular bacteria such as *Salmonella* or BCG, as a vaccine vector due to its unusual life cycle. As facultative intracellular bacteria, *Listeriae* are taken up primarily by APCs such as macrophages and DCs and enter their phagosomes. The majority of bacteria are killed and digested in the lysosomal compartment *in vivo*, thus targeting the antigens to the MHC class II pathway for antigen processing and cell surface presentation. At the same time, some of the bacteria will escape into the cytosol through the actions of the LLO protein. Antigens from *Listeria* that multiply in the cytosol are presented in an MHC class I-restricted manner to T cells, allowing the expansion of CD8<sup>+</sup> T cells, which are necessary for clearance of virally infected host cells as well as being important for direct killing of tumour cells. The MHC class II antigen presentation of bacteria-derived antigens in the lysosome induces a CD4<sup>+</sup> T-cell response, which is also necessary for a robust, cell-mediated immune response.

LLO, produced by *Listeria monocytogenes*, is a member of the cholesterol-dependent cytolysin (CDC) family of toxins. It is the main virulence factor in the pathogenesis of *listeriosis*, allowing the bacteria to escape from host-cell phagosomes to replicate in the cytoplasm without killing the host cells (Birmingham, Canadien et al. 2008). The use of a recombinant *L. monocytogenes* strain in which expression of LLO was placed under the control of an Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG)-inducible promoter (Dancz, Haraga et al. 2002) confirmed that primary phagosomal escape and intracellular growth of *L. monocytogenes* was strictly dependent upon expression of LLO. Mutants of *L. monocytogenes* lacking LLO-secretion are generally non-virulent (Kayal and Charbit 2006).

The advantage of LLO incorporation into vaccine resides in the fact that, after degradation of the vaccine strain within the phagolysosome, target antigens are released into the cytosol for endogenous processing and presentation for stimulation of CD8<sup>+</sup> effector T cells. Using this principle, Bouwer et al (Brockstedt, Bahjat et al. 2005) suggested that this approach could be used against intracellular bacterial pathogens. Ikonomidis et al had first shown the use of *L. monocytogenes* to deliver Influenza antigen to the class I pathway and induced antigen-specific CTLs *in vivo* (Pan, Ikonomidis et al. 1995). Further evidence is the preferential skewing differentiation of antigen-specific T cells into Th1 cells and inhibit Th2 (Yamamoto, Kawamura et al. 2005).

### 1.3.2 Recombinant *E. coli* expressing LLO as protein delivery vector

Non-pathogenic bacteria, *E. coli*, harbouring plasmid encoding LLO, without the secretion signal, have been proposed as an alternative to *Listeria*. Higgins et al have shown that *E. coli* expressing cytoplasmic recombinant LLO can efficiently deliver co-expressed proteins to the cytosol of macrophages (Higgins, Shastri et al. 1999). In this model, a large enzymatically active protein was delivered to the cytosol (Figure 3).



**Figure 3. Antigen presentation pathway involved in OVA and LLO-expressing *E. coli*.**

Lysosomal digestion of the phagocytosed *E. coli* caused the release of encoded antigen (OVA) and pore-forming of lysosome by LLO. As a result, the digested bacterial products and OVA were transferred into the cytosol after disruption of the lysosomal membrane by LLO. The digested bacterial products or PAMPs can interact with cytosolic receptor or Toll-like receptor and OVA can be degraded and processed through proteasome/endoplasmic reticulum and presented onto cell surface in conjunction with MHC class-I complex.

Furthermore, they demonstrated that the *E. coli*-LLO system is very efficient for delivery of ovalbumin (OVA) to the MHC class I pathway for antigen processing and presentation, greater than 4 logs compared with *E. coli* expressing OVA alone (Higgins, Shastri et al. 1999).

Previous experiments had demonstrated that vaccination with *E. coli*-LLO-pulsed DCs expressing OVA generated OVA-specific CTLs and tumour protection in a murine tumour model (Radford, Higgins et al. 2002). Direct injection of *E. coli*-LLO/OVA resulted in more dramatic anti-tumour immunity than vaccination with pulsed DCs. Also, subcutaneous injection of paraformaldehyde-fixed *E. coli*-LLO provided an additional safety feature without compromising vaccine efficacy. Further experiments have shown that fixed *E. coli*-LLO expressing the well-characterised human melanoma antigen, MART1, efficiently deliver the HLA-A2-restricted MART1<sub>27-35</sub> epitope for processing and presentation on human MoDCs, suggesting the potential of this system as a novel strategy for human tumour immunotherapy (Radford, Jackson et al. 2003).

### **1.3.3 Advantage of the *E. coli*-LLO model in antigen processing**

The *E. coli*-LLO system only requires expression of the target protein in the bacteria. High levels of protein can be delivered to the cytosol of virtually all cells in culture. The level of protein produced and ultimately delivered to the cytosol of macrophages can be controlled. By expressing invasive determinants from other bacterial species, the *E. coli* could be modified to enter cells other than macrophages or antigen-presenting cells which are naturally phagocytic. Expression of Invasin could be used for delivery of proteins to epithelial cells (Critchley, Jezard et al. 2004; Critchley-

Thorne, Stagg et al. 2006). Invasin binds to  $\beta$ 1-integrin expressed at the surface of mammalian cells. This binding to  $\beta$ 1-integrin is necessary and sufficient for entry of the whole bacterium into the mammalian cell (Marra and Isberg 1996). It has been shown that diaminopimelate (DAP)-minus *E. coli* expressing invasin from *Yersinia pseudotuberculosis* has the ability to invade cultured mammalian cells and lyse within the phagosome (Grillot-Courvalin, Goussard et al. 1998).

The *E. coli*-LLO system provides another advantage. Full-length tumour-antigen can be expressed safely in bacteria as opposed to the host cell. This has an added advantage in that antigenic epitopes do not need to be defined and treatment is less likely to be restricted to patients of specific HLA haplotypes. Many tumour antigens have already successfully been expressed as recombinant proteins in *E. coli*, demonstrating the simplicity of this technique. In addition, the injected bacterial cell components, PAMPs, also can potentiate the immune response against the antigen and thus play an adjuvant role in the treatment.

#### **1.4 AIMS OF THIS STUDY**

In previous studies in the host laboratory, *E. coli*-LLO/OVA have a superior effect in tumour protection. My main research aimed at unraveling the immune mechanism induced by the bacterial vaccination. In addition, chicken ovalbumin was exploited as a model tumour antigen. A key question was whether the previously described efficacy of *E. coli*-LLO can be obtained against a real tumour antigen– with clinical relevance and to assess the autoimmunity to normal tissues.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 BACTERIAL STRAINS

The *E. coli* strain MC4100 (DE3) used in our study was a gift from Prof. DE Higgins, Harvard Medical School, Boston, USA. It is a derivative of the K12 *E. coli* strain, harbouring the DE3 bacteriophage, which contains the T7 RNA polymerase gene that allows Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG)-inducible expression of gene under T7 promoter control. *E. coli*-LLO harbours the plasmid pDP-E3615 which encodes listeriolysin O lacking its secretion signal under the control of the constitutive *tet* promoter.

The cDNA of WT1 protein was amplified from a mouse cDNA lacking the two alternative splicing donor sites (17aa-/KTS-) by PCR. The 5' primer used as 5'-ATG CAC TCC TTC ATC AAA CAG GAG CCCA-3'. The 3'-primer used was 3'-TCA AAG CGC CAC GTG GAG TTT GGTC-5'. The PCR product was cloned using TOPO CT cloning kit (Invitrogen). The sequence of the PCR product was analysed by restriction enzyme digestion and capillary electrophoresis sequencer 3700 (Applied Biosystems), and shown to be identical to the previously published mouse WT1 gene sequence. The cloned WT1 gene expressed a truncated version of WT1 (67 amino acids at the N-terminus domain are missing), as it was impossible in our hands to express the whole coding sequence in bacteria (work performed by Dr. Josianne Nitcheu). Plasmid pCRT7/CT which encodes WT1 cDNA under the control of IPTG-inducible T7 phage promoter was transformed into DE3 containing or not the plasmid encoding LLO (*E. coli*-LLOWT1 or *E. coli*-WT1).

## **2.2 EXPRESSION OF TARGET PROTEINS BY IPTG**

Bacteria containing dual plasmids were cultured in medium containing Ampicillin (50 µg/mL) and Chloramphenicol (40 µg/mL). Single colonies of *E. coli*-WT1 or *E. coli*-LLO/WT1 were inoculated from an LB agar plate into 10 ml of LB medium containing appropriate antibiotics and grown overnight to stationary phase at 30°C with aeration and shaking. Starter cultures were diluted into 500 ml of LB medium in 1L flasks and continued to grow for 2 h with aeration and shaking at 30°C. Protein expression was induced by the addition of IPTG to 1 mM, and growth continued for an additional 4 h until cultures reached an OD<sub>600</sub> of 0.7~1.0. The bacterial concentration from cultures was estimated by measuring the optical density (O.D.) at 600 nm. Serial dilution assays to determine the bacterial number showed that an OD<sub>600</sub> 0.8 corresponds to a concentration of 10<sup>9</sup>/mL MC4100 (DE3) *E. coli* and OD<sub>600</sub> 1.0 corresponds to a concentration of 5x10<sup>8</sup>/mL *E. coli* WT1 or *E. coli* LLO/WT1. The bacteria were harvested by centrifugation at 4000 rpm for 10 minutes, and the resulting pellet was resuspended to the appropriate concentration in LB broth containing 15% Glycerol and stored at –80°C until further use for vaccination.

## **2.3 CONFIRMATION OF PROTEIN EXPRESSION IN BACTERIA BY WESTERN BLOT**

Equivalent numbers of bacteria (10<sup>8</sup>) were centrifuged (14,000 g) for 1 min and washed once with phosphate-buffered saline (PBS). Washed pellets were re-suspended in Laemmli buffer (250 mM Tris pH6.8, 5% Sodium dodecyl sulphate (SDS), 20% glycerol, 0.01% bromophenol blue) and boiled for 5 min, and total



cellular protein was analysed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie brilliant blue or specific primary/secondary antibodies in a Western Blot. For WT1 expression, (primary) mouse anti-WT1 Ab (1/1000, DAKO, U.S.) and (secondary) rabbit anti-mouse Ig- horseradish peroxidase (HRP) (1/2000, DAKO, U.S.) were used. For LLO expression, (primary) rabbit anti-LLO Ab (1/2000, Diatheva, Italy) and (secondary) anti-rabbit Ig-HRP (1/1000, GE Healthcare, U.K.) were used. The signal was revealed by incubating the membrane with ECL detection agent (Amersham Biosciences, U.K.) according to the manufacturer's instructions.

## 2.4 PEPTIDES SYNTHESIS

Specific peptides were synthesised by the peptide synthesis laboratory of Cancer Research UK (London LIF, UK). The quality of the peptides was assessed by HPLC analysis and the molecular weight confirmed by mass spectrometry. All peptides were dissolved in distilled-water at 1 mM concentration and kept at -20°C. When required, 0.5-1% DMSO was added. Specific peptide sequences are shown in Table 2.

**Table 2 Specific peptide sequence**

Peptide (sequence)	Sequence
OVA <sub>257-264</sub>	SIINFEKL
pWT119 (pWT <sub>119-127</sub> )	QASSGQARM
pWT126 (pWT <sub>126-134</sub> )	RMFPNAPYL
pWT130 (pWT <sub>130-138</sub> )	NAPYLPSCL
pWT235 (pWT <sub>235-243</sub> )	CMTWNQMNL
pWT423 (pWT <sub>423-431</sub> )	KKFARSDEL

The WT1 peptide library was generated from the human WT1 sequence (Minotopes, UK). It consisted of 106 15-mer peptides overlapping by 11 amino acids, spanning the length of the WT1 sequence (Table 3). The peptides were dissolved in a minimal volume of DMSO with 2mM DTT (Fischer Scientific, Loughborough) where necessary. The peptides were then resuspended in water to make 5mM stock solution and stored at -80°C. For experimental use individually the peptides were diluted with media to an intermediate concentration of 100 µM.

**Table 3. Human WT1 peptide library**

Peptide number	Aminoacid Sequence	From	To	Peptide number	Aminoacid Sequence	From	To
1	MGSDVRDLNALLPAV	1	15	54	QALLLRTPYSSDNLY	213	227
2	VRDLNALLPAVPSLG	5	19	55	LRTPYSSDNLYQMTS	217	231
3	NALLPAVPSLGGGGG	9	23	56	YSSDNLYQMTSQLEC	221	235
4	PAVPSLGGGGGCALP	13	27	57	NLYQMTSQLECMTWN	225	239
5	SLGGGGGCALPVSGA	17	31	58	MTSQLECMTWNQMNL	229	243
6	GGGCALPVSGAAQWA	21	35	59	LECMTWNQMNLGATL	233	247
7	ALPVSGAAQWAPVLD	25	39	60	TWNQMNLGATLKGHS	237	251
8	SGAAQWAPVLDFAFP	29	43	61	MNLGATLKGHSTGYE	241	255
9	QWAPVLDFAFPPGASA	33	47	62	ATLKGHSTGYESDNH	245	259
10	VLDFAFPPGASAYGSL	37	51	63	GHSTGYESDNHTTPI	249	263
11	APPGASAYGSLGGPA	41	55	64	GYESDNHTTPILCGA	253	267
12	ASAYGSLGGPAPPPA	45	59	65	DNHTTPILCGAQYRI	257	271
13	GSLGGPAPPPAPPPP	49	63	66	TPILCGAQYRIHTHG	261	275
14	GPAPPPAPPPPPPPP	53	67	67	CGAQYRIHTHGVFRG	265	279
15	PPAPPPPPPPPHSF	57	71	68	YRIHTHGVFRGIQDV	269	283
16	PPPPPPPHSFQKQE	61	75	69	THGVFRGIQDVRRVP	273	287
17	PPPHSFQKQEPSWG	65	79	70	FRGIQDVRRVPGVAP	277	291
18	HSFIKQEPSWGGAEP	69	83	71	QDVRRVPGVAPTLVR	281	295
19	KQEPSWGGAEPHEEQ	73	87	72	RVPGVAPTLVRSASE	285	299
20	SWGGAEPHEEQCLSA	77	91	73	VAPTLVRSASETSEK	289	303
21	AEPHEEQCLSAFTVH	81	95	74	LVRSASETSEKRPFM	293	307
22	EEQCLSAFTVHFSGQ	85	99	75	ASETSEKRPFMCAYP	297	311
23	LSAFTVHFSGQFTGT	89	103	76	SEKRPFMCAYPGCNK	301	315
24	TVHFSGQFTGTAGAC	93	107	77	PFMCAYPGCNKRYF	305	319
25	SGQFTGTAGACRYGP	97	111	78	AYPGCNKRYFKLSHL	309	323
26	TGTAGACRYGPFGPP	101	115	79	CNKRYFKLSHLQMHS	313	327
27	GACRYGPFGPPPPSQ	105	119	80	YFKLSHLQMHSRKHT	317	331
28	YGPFGPPPPSQASSG	109	123	81	SHLQMHSRKHTGEKP	321	335
29	GPPPPSQASSGQARM	113	127	82	MHSRKHTGEKPYQCD	325	339
30	PSQASSGQARMFPNA	117	131	83	KHTGEKPYQCDFKDC	329	343
31	SSGQARMFPNAPYLP	121	135	84	EKPYQCDFKDCERRF	333	347
32	ARMFPNAPYLPSCLE	125	139	85	QCDFKDCERRFSRSD	337	351
33	PNAPYLPSCLESQPA	129	143	86	KDCERRFSRSDQLKR	341	355
34	YLPSYLESQPAIRNQ	133	147	87	RRFSRSDQLKRHQRR	345	359
35	CLESQPAIRNQGYST	137	151	88	RSDQLKRHQRRHTGV	349	363
36	QPAIRNQGYSTVTFD	141	155	89	LKRHQRRHTGVKPFQ	353	367
37	RNQGYSTVTFDGTPS	145	159	90	QRRHTGVKPFQCKTC	357	371
38	YSTVTFDGTPSYGHT	149	163	91	TGVKPFQCKTCQRKF	361	375
39	TFDGTPSYGHTPSHH	153	167	92	PFQCKTCQRKFSRSD	365	379
40	TPSYGHTPSHHAAQF	157	171	93	KTCQRKFSRSDHLKT	369	383
41	GHTPSHHAAQFPNHS	161	175	94	RKFSRSDHLKTHTRT	373	387
42	SHHAAQFPNHSFKHE	165	179	95	RSDHLKTHTRTHTGK	377	391
43	AQFPNHSFKHEDPMG	169	183	96	LKTHTRTHTGKTSEK	381	395
44	NHSFKHEDPMGQQGS	173	187	97	TRTHTGKTSEKPFSC	385	399
45	KHEDPMGQQGSLGEQ	177	191	98	TGKTSEKPFSCRWPS	389	403
46	PMGQQGSLGEQQYSV	181	195	99	SEKPFSCRWPSQQKK	393	407
47	QGSLGEQQYSVPPPV	185	199	100	FSCRWPSQQKKFARS	397	411
48	GEQQYSVPPPVYGCH	189	203	101	WPSCQKKFARSDELV	401	415
49	YSVPPPVYGCHTPTD	193	207	102	QKKFARSDELVRHHN	405	419
50	PPVYGCHTPTDSTCTG	197	211	103	ARSDDELVRHHNMHQR	409	423
51	GCHTPTDSTCTGSQAL	201	215	104	ELVRHHNMHQRNMTK	413	427
52	PTDSTCTGSQALLRT	205	219	105	HHNMHQRNMTKLQLA	417	431
53	CTGSQALLLRTPYSS	209	223	106	HNMHQRNMTKLQLAL	421	435

## **2.5 CELL LINES**

RMA (K<sup>b</sup>D<sup>b</sup>), their derivative RMA-S, MBL2, MBL2-WT1, TRAMP-C cell lines were kindly provided by Prof. HJ Stauss (Royal Free Hospital, London). RMA is a murine thymoma cell line, and RMA-S is a TAP-deficient variant that was obtained by mutagenesis of RMA followed by selection of MHC class I-loss variants. MBL2 is a leukaemia cell line isolated from M-MuLV-infected C57BL/6 mouse. The prostate cancer cell line TRAMP-C (kind gift from Dr. N. Greenberg) was isolated from transgenic mice expressing SV40 large T antigen in the prostate tissue. RMA-WT1 and MBL2-WT1 cells were transfected with a plasmid containing the WT1 gene under the control of a CMV promoter. RMA and RMA-S cells were cultured in complete RPMI containing 10% Fetal calf serum (FCS). MBL2 cells were cultured in complete RPMI containing 50 µM β-Mercaptoethanol and 10% FCS. Transfected cells (MBL2-WT1 and RMA-WT1) were maintained in the presence of appropriate concentration of G418 (Geneticin, Invitrogen). TRAMP-C cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Invitrogen) supplemented with 10% FCS, 10% Nu-Serum<sup>TM</sup> (BD Biosciences), 5 µg/mL insulin, and 0.01 nM testosterone. B16-OVA murine melanoma cells were provided by Cancer Research UK Cell Services (LIF, London) and grown in DMEM, 10% FCS and G418 (400 µg/mL). All cell lines were routinely tested and found to be free of mycoplasma.

## **2.6 IMMUNISATION WITH PEPTIDE**

Mice were immunised subcutaneously in the flank with 100 µg of peptide diluted in phosphate-buffered saline emulsified 1:1 with Incomplete Freuds adjuvant (IFA)

(Sigma). Mice were immunised 3 times at one week intervals. In some experiments mice were also injected with fixed *E. coli*-LLO resuspended in PBS ( $10^8$ /mouse) and 100 µg of peptide.

## 2.7 ANIMALS

C57/BL6 mice ( $K^bD^b$ ) from Harlan (Harlan Ltd, Bicester, UK) were used at 6-8 weeks of age (weight 18-20 gm) for immunisation and as a source of accessory cells for *in-vitro* CTL stimulation. They were maintained in a specific pathogen-free containment facility at Queen Mary University of London Biological Resources Unit (Charterhouse Square). Experiments were conducted after appropriate ethical approval and licensing was obtained in accordance with the United Kingdom "Guidance on the operation of animals (Scientific Procedure) Act 1986". The mice were subcutaneously injected 3 times at one week intervals with  $10^8$  Paraformaldehyde-fixed bacteria resuspended in 100 µL PBS. One week after the last vaccination, syngeneic TRAMP-C cells ( $10^6$ /mouse), MBL2-WT1 cells ( $5 \times 10^6$ /mouse), or B16-OVA cells ( $5 \times 10^5$ /mouse) were injected subcutaneously to each mouse. The tumour size was monitored and measured in 2-dimensions ( $\text{mm}^2$ ). The dose of tumour cells inoculated was optimised in preliminary experiments.

## 2.8 PREPARATION OF BMDCS

Total mononuclear cells were harvested by flushing of the bone marrow content of the femur and tibia of C57BL/6 mice. Cells were cultured in RPMI-1640 at  $10^6$  cells/mL with GM-CSF (5 ng/mL). The culture was refed with fresh medium and G-

CSF on 3<sup>rd</sup> day and all the non-attached cells were collected after 5 days as for the BMDCs. For loading,  $1 \times 10^8$  of each bacteria were added to  $1 \times 10^6$  BMDCs in a volume of 1 ml in polypropylene tubes in RPMI-1640 medium supplemented with 10% FCS. After 1 h of incubation, Ag-pulsing medium was decanted by several washes before the BMDCs were used for assay.

## **2.9 IN VITRO CTL STIMULATION AND CHROMIUM-51 RELEASE (CTL) ASSAY**

Splenocytes from immunised mice ( $3 \times 10^6$  cells/mL) were stimulated *in vitro* with LPS-stimulated syngeneic spleen cells (splenic blasts,  $10^6$  cells/mL) loaded with specific peptides. Spleen cell blasts were prepared by stimulating B6 spleen cells ( $1.5 \times 10^6$  cells/mL) with 25  $\mu\text{g/mL}$  LPS (Sigma) and 7  $\mu\text{g/mL}$  dextran sulphate (Sigma) (Sette, Vitiello et al. 1994). Three days after activation cells were irradiated (30 cGy) and incubated with peptide ( $10^6$  cells/100  $\mu\text{L}$  irradiated blasts in 100  $\mu\text{M}$  peptide concentration, 1 hour in 37°C before mixing with spleen cells from immunised mice. Stimulated cells were collected 5 or 6 days later for the effector cells in CTL assay.

CTL cultures were analysed in standard  $^{51}\text{Cr}$  release assay after *in-vitro* stimulation. A standard chromium release assay was used (Dahl, Beverley et al. 1996) and  $10^6$  target cells were incubated at 37°C with 50  $\mu\text{Ci}$   $^{51}\text{Cr}$  (Amersham Biosciences) for 60 minutes in the presence of specific peptides. Cells were washed 3 times and resuspended in RPMI with 5% FCS. When peptide-loaded RMA-S cells were used as target cells, they were temperature induced (26°C) overnight before the assay. Assays were performed by mixing  $5 \times 10^3$   $^{51}\text{Cr}$ -labeled cells with varying dilutions of effector cells (in 100  $\mu\text{L}$ ) and incubated at 37°C for 4h. At the end of the incubation, 100  $\mu\text{L}$  of the supernatant was analysed on a  $\gamma$ -counter (1470 Wizard Gamma

Counter, Perkinelmer, UK). CTL were always tested against the specific peptide as well as an irrelevant control peptide. All assays were performed at least in triplicate. Specific cytotoxicity was calculated using the formula: ((experimental release-spontaneous release) / (maximal release-spontaneous release)) x 100%.

## **2.10 ANTIBODIES AND *IN-VIVO* DEPLETION OF T CELL SUBSETS**

The anti-CD4 (GK1.5), anti-CD8 (YTS 169.4), and PLTY-1 (isotype control) mAbs were purified from relevant hybridomas (Cancer Research-UK). Anti-CD25 mAb (clone PC61) and its isotype control (rat IgG1) were purchased from BioExpress (West Lebanon, NH). On days 0, 7 and 14, C57BL/6 mice were vaccinated subcutaneously (s.c.) with  $10^8$  of either bacterium. Depletion of CD4<sup>+</sup> was achieved at the time of CD8<sup>+</sup>T cell priming by intra-peritoneal (i.p.) administration of 300 µg of GK1.5 on days -5 and 10. CD8 + T cells were depleted by *in-vivo* administration of 300 µg of YTS 169.4 depleting mAbs on days 10 and 17. Control mice were treated with the same doses of the relevant isotype control mAbs or PBS. To deplete CD25<sup>+</sup> cells, a total of 400 µg of PC61 Ab was injected i.p. on day -1. Optimal conditions of depletion of these T cell subsets were determined according to previous publications (Rice and Bucy 1995; Noort, Benner et al. 1996; Haeryfar, DiPaolo et al. 2005), and were shown to totally delete CD25<sup>+</sup> cells in peripheral blood and significantly diminish the percentage of CD25<sup>+</sup> in the spleen and LN for at least 10 days following the injection.

## **2.11 TREG, CD4<sup>+</sup>, OR CD8<sup>+</sup> PURIFICATION AND FLOW CYTOMETRIC ANALYSIS**

Single-cell suspensions were obtained from the spleens of naïve or vaccinated mice. CD4<sup>+</sup> cells were negatively selected and fractionated into CD4<sup>+</sup>CD25<sup>low</sup> and CD4<sup>+</sup>CD25<sup>high</sup> subsets by magnetic antibody cell sorting (MACS, Miltenyi Biotech, Germany), using PE-labeled anti-CD25 mAb followed by anti-PE microbeads, according to the manufacturer's instructions. The purity of cells was confirmed by FACS analysis, and > 90% of the cells were shown to be either CD25<sup>low</sup> or CD25<sup>high</sup>. CD4<sup>+</sup> or CD8<sup>+</sup> subsets were selected positively by anti-CD4 or anti-CD8 microbeads according to the manufacturer's instruction (MACS, Miltenyi Biotech, Germany). Enriched CD4<sup>+</sup> and CD8<sup>+</sup> subset purity was analysed by flow cytometry.

## **2.12 ELISPOT ASSAYS**

Ninety-six-well ELISpot plates (Millipore) were coated with 100 µl/well of 15 µg/mL purified anti-mouse IFN-γ mAb (BD Bioscience) overnight at 4°C. Plates were washed 5 times with PBS before addition of 8X10<sup>5</sup> splenocytes in triplicate wells and 10 µM of peptide. Concanavalin A was used as a positive control. After 20 h incubation at 37°C in 5% CO<sub>2</sub>, plates were developed by incubating with 50 µL per well of biotinylated anti-IFN<sub>γ</sub> (BD Biosciences) at 1µg/mL in PBS for 2h at 37°C. Streptavidine alkaline phosphatase (100 µL) (Caltag, UK) was added to each well after 5 washes and incubated for 1 h at RT. The plate was developed using 100 µL of alkaline phosphatase conjugate substrate (BioRad). Spots were counted by an automated ELISpot reader and a response was considered positive when spot numbers in triplicate assays in the presence of the specific peptide significantly



exceeded the cutoff value, corresponding to the number of non specific spots in the presence of irrelevant peptide.

### 2.13 LYMPHOCYTE PROLIFERATION ASSAY

Splenocytes from 2 bacteria-vaccinated mice of each group (*E. coli*, *E. coli* WT1, and *E. coli* LLO/WT1) were used as responder cells ( $2 \times 10^5$  cells/well) and mixed with irradiated splenocytes as feeder cells from naïve mice ( $2 \times 10^5$  cells/well). These cells were stimulated with various peptides (20  $\mu$ M with/without Interleukin-2), which were selected from previous ELISpot screening of whole WT1 peptide library. The plates were incubated at 37°C in 5% CO<sub>2</sub> for 72 hours and pulsed with 1  $\mu$ Ci/well of <sup>3</sup>H-Thymidine (Amersham Biosciences). The radioactivity incorporated into DNA, which correlates with the cell proliferation, was measured in a liquid scintillation counter after harvesting the cell cultures onto the glass fiber filters 18 hours after adding <sup>3</sup>H-Thymidine. All assessments of proliferative responses were carried out at least in six replicate, and the results corresponded to the mean values.

For proliferatory/regulatory assays,  $10^5$  CD4+CD25<sup>high</sup> regulatory cells or CD4+CD25<sup>low</sup> responder cells or both were cultured in RPMI medium containing 10 % FCS, 50  $\mu$ M 2-mercaptoethanol with 0.5  $\mu$ g/mL anti-CD3 Ab (purified anti-CD3, Pharmingen) in the presence of  $2 \times 10^5$  naïve irradiated splenocytes. The cells were cultured for 5 days and proliferation was measured by adding 1 $\mu$ Ci of <sup>3</sup>H-Thymidine (Amersham, UK) to each well for the last 18hr of the culture period. The cells were harvested and the <sup>3</sup>H-Thymidine incorporation was determined. The suppression of Tconv proliferation by Treg (% inhibition) is calculated as [Proliferation (Tconv only) - Proliferation (Treg+Tconv)] x 100% / Proliferation (Tconv only).

## **2.14 RNA EXTRACTION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION**

Total cellular RNA was prepared from  $10^7$  cells in TRIzol reagent (Invitrogen, Carlsbad, CA). Following chloroform extraction, total RNA was precipitated in isopropanol, washed with ethanol, suspended in an RNase-free solution by RNeasy Mini kit (Qiagen), and stored at  $-80^{\circ}\text{C}$  until used for cDNA synthesis. Total RNA was quantified spectrophotometrically based on an absorbance at 260 nm of one equal to an RNA concentration of  $40\ \mu\text{g}/\text{mL}$ .

RNA was converted into cDNA with RT-PCR kit (Taq Man Reverse Transcription Agent, Applied Biosystems). RNA integrity was verified by RNA 6000 Nano LabChip (Agilent Technologies) and quantified by a spectrophotometer according to the absorbance at 260 nm and 280 nm. Up to  $2\ \mu\text{g}$  of total RNA were processed in  $30\ \mu\text{L}$  volume, with  $94\ \text{u}$  MultiScribe™ Reverse Transcriptase (Applied Biosystems) using random hexamer primers. In brief,  $1\ \mu\text{g}$  of total RNA was mixed with  $1.5\ \mu\text{L}$  random hexamer primers ( $50\ \mu\text{M}$ ),  $6\ \mu\text{L}$  deoxynucleotide mix ( $2.5\ \text{mM}$ ),  $3\ \mu\text{L}$  Taq Man RT buffer ( $10\text{X}$ ),  $6.6\ \mu\text{L}$   $\text{MgCl}_2$  ( $25\ \text{mM}$ ), and  $0.6\ \mu\text{L}$  RNase inhibitor ( $20\ \text{u}/\text{L}$ ). The RT programme was set as:  $25^{\circ}\text{C}$  for 10 min,  $37^{\circ}\text{C}$  for 60 min,  $95^{\circ}\text{C}$  for 5 min for heat inactivation, and finally stopped at  $4^{\circ}\text{C}$ .

For standard curves, a 10-fold serial dilution of RNA of the human leukaemic cell line K562 was performed in DEPC-treated water, ranging from  $1 \times 10^{-1}$  to  $1 \times 10^{-5}$ . Every dilution step was processed in duplicate and all samples were tested in triplicate.

Real-time quantitative PCR was performed using the ABI Prism 7500 detection system (Applied Biosystems) with SYBR® Green RT-PCR Reagents. The cDNA was

serially diluted and WT1 mRNA was amplified using the appropriate oligonucleotide primers by PCR for 50 cycles in PCR machine. Primer for WT1 was: sense, 5'-TAC CCA GGC TGC AAT AAG AGA TAT TTT AAG-3', antisense, 5'-CCT TGG GTG TCTTTT GAG CTG GTC-3'. The reaction was performed in 20  $\mu$ L volume. For each reaction, cDNA (equivalent of 100 ng RNA) was processed to detect WT1 expression. The concentration of forward and reverse primers was 750 nM. The cyclor programme consisted of an initial 94°C step for 3 min followed by denaturation at 72°C for 10 min, The q-PCR includes further 50 cycles with one denaturation (94°C, 45 s), annealing step (56°C, 45 s), and 72°C, 60 s. The expected size for the WT1 PCR product was 147 bp. To assure that the PCR products were not being assayed on the plateau of the amplification curve, all cDNA samples were amplified at three 10-fold dilutions. This procedure allowed quantitative comparisons to be made of the levels of cDNA present in different samples.

## **2.15 MEASUREMENT OF BINDING STABILITY OF WT1 PEPTIDES FOR MHC**

### **CLASS-I MOLECULES (H2-D<sup>b</sup>/K<sup>b</sup>)**

The ability of synthetic WT1 peptides to bind to H2-D<sup>b</sup> or H2-K<sup>b</sup> class I molecules was detected by using the leukaemia cell line RMA-S, as described previously (Ljunggren, Stam et al. 1990). In brief, RMA-S cells were cultured for 12 hours at 26°C in complete RPMI supplemented with 10% FCS. A total of 10<sup>6</sup> RMA-S cells were added to each well of a 96-well plate and incubated either alone or with the designated peptide (100  $\mu$ M) for 2 hours at 37°C. Cells were then washed once and stained with fluorescein isothiocyanate (FITC)-conjugated anti-D<sup>b</sup> or anti-K<sup>b</sup> antibody (Pharmingen, San Diego, CA). Labeled cells were washed twice, resuspended, and fixed in 500  $\mu$ L phosphate-buffered saline with 1% paraformaldehyde and were

analyzed for fluorescence intensity in a flow cytometer (FACScalibur; Becton Dickinson). The percentage of increase of D<sup>b</sup> or K<sup>b</sup> molecules on the surface of the RMA-S cells was measured by increased mean fluorescence intensity of cells incubated with peptide compared with that of cells incubated in medium alone or OVA<sub>257-264</sub> peptide, SIINFEKL.

## **2.16 ANALYSIS OF THE ANTIBODY RESPONSE IN WT1 VACCINATED**

### **ANIMALS**

The recombinant WT1 protein (Santa Cruz Biotechnologies) 1.5 µg per lane was resolved on a 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, UK) by semi-dry transfer. The membrane was then blocked in PBS containing 0.01% Tween 20 and 5% nonfat dry milk overnight at 4°C and incubated first with serum from vaccinated mice (1:100 in blocking solution) for 2 hours at room temperature and then with HRP-conjugated rabbit anti-mouse immunoglobulin (1:2000 in blocking solution) for 1 hour at room temperature. The signal was revealed by incubating the membrane with ECL detection agent (Amersham) according to the manufacturer's instructions.

## **2.17 TREG RNA MICROARRAY ANALYSES**

### **2.17.1 Total RNA extraction**

Total RNA was extracted from purified CD4<sup>+</sup>CD25<sup>+</sup> cells (~10<sup>6</sup> cells in each group) using Qiagen RNeasy micro-columns according to the manufacturer's protocol. RNA

yields were determined spectrophotometrically at 260 nm and RNA integrity checked by capillary electrophoresis using an Agilent 2100 bioanalyzer (Agilent, South Queensferry, UK).

### **2.17.2 Production of fragmented labeled cRNA and Array hybridisation**

First-strand cDNA was synthesised from 1 µg total RNA by incubation (42°C, 1 hr) in a 20-µL reaction volume containing 2.5 mM T7-(dT)<sub>24</sub> primer, 50 mM Tris-HCl pH 8.3, 75 mM KCL, 3 mM MgCl<sub>2</sub>, 10 mM DTT and 500 mM dNTP, 10 U/µL Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand cDNA was synthesized directly by adding 91 µL RNase-free water, 30 µL 5 times second-strand reaction buffer (Invitrogen), 3 µL 10 mM dNTP, 1 µL *Escherichia coli* DNA ligase (10 U/µL), 4 µL *E. coli* DNA polymerase I (10 U/µL), 1 µL *E. coli* RNase H (2 U/µL) followed by incubation (2 hr, 16°C). The ends of the double-stranded cDNA were polished using T4 DNA polymerase (20 U, 5 min, 16°C). The cDNA was purified and concentrated by phenol/chloroform extraction and ethanol precipitation.

Generation of biotin-labeled RNA was accomplished by *in-vitro* transcription with the T7 RNA polymerase using the IVT kit (Affymetrix). Biotin-labeled cRNA was subsequently purified from the transcription reaction using the RNeasy system (Qiagen, Hilden, Germany). Hybridisation of biotin-labeled cRNA to GeneChip Mouse Genome 430 2.0 Array, washing, staining, and scanning were performed according to the protocols published by the manufacturer (Affymetrix). The GeneChip Mouse Genome 430 2.0 Array comprises more than 40,000 transcripts on a single array probe set.

### **2.17.3 Data processing**

Samples were profiled using the Affymetrix Mouse Genome 430 2.0 Array. Data were analysed using Bioconductor (<http://www.bioconductor.org/>) packages within the open source R statistical environment ([www.r-project.org](http://www.r-project.org)). We used the QC metrics recommended by Affymetrix and the probe level models as well as boxplots and intensity histograms for quality assessment. After background correction and normalisation by robust multi-array analysis (RMA), we applied a filter using the standard deviation (SD) of gene expression values to select the top 6000 genes on the array. We used Limma (Wettenhall and Smyth 2004) for differential expression analysis. Briefly, Limma fit a linear model to the expression data for each probe. Empirical Bayes is used to obtain information across genes making the analyses stable even for experiments with small number of arrays. We set a double cutoff of false discovery rate (FDR) < 0.05 and a fold change  $\geq 2$ .

### **2.17.4 Meta-analysis**

With the assistance from Dr. Claude Chelala, we performed a meta-analysis using the raw data from Gene Expression Omnibus data <http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSM44980>. We applied the same analysis pipeline described above.

### **2.17.5 Verification of the expression patterns for the selected genes by RT-PCR**

RNA was converted into cDNA with RT kit (Taq Man Reverse Transcription Agent, Applied Biosystems) as described in **2.13**. The cDNA quantity of both samples (*E. coli* and *E. coli*-LLO) was adjusted by housekeeping genes (6S and GAPDH).

Selected genes were amplified using the appropriate oligonucleotide primers by polymerase chain reaction (PCR) for 30 cycles in a Mendel PCR machine (Gene Legends Ltd, Ringmer, UK) in the thermal programme of: initial 94°C-3 min, followed by 30 cycles of 94°C-30 sec, 60°C-45 sec, and 68°C-45 sec, then final denaturation 68°C-7min. Specific amplified DNA products were migrated on ethidium bromide-containing agarose gel and visualised under UV light.

**Table 4. List of primers for RT-PCR verification**

Gene		Forward Primer	Reverse Primer
S100 A8	NM_013650	GGAAATCACCATGCCCTCTA	TGGCTGTCTTTGTGAGATGC
S100 A9	NM_009114	TCATCGACACCTTCCATCAA	GTCCTGGTTTGTGTCCAGGT
Lipocalin-2	X14607	GCCTCAAGGACGACAACATCAT	GGCTCTCTGGCAACAGGAAAG
NGP	NM_008694	CCACTCCGCCTTCTAGTCAG	AGGTCAAGGTCAGGGAGGTT
CCR1	AV231648	GTTGGGACCTTGAACCTTGA	CCCAAAGGCTCTTACAGCAG
TGFβi	NM_009369	TGATAAGAGGGGACGGTTTG	ATTGGTGGGAGCAAAAACAG
IL1β	NM_008361	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCTG
IL17	NM_010552	TCCAGAAGGCCCTCAGACTA	AGCATCTTCTCGACCCCTGAA
CXCL2	NM_009140	AAGTTTGCCTTGACCCTGAA	AGGCACATCAGGTACGATCC
IL-22	NM_016971	ATACATCGTCAACCGCACCTTT	AGCCGGACATCTGTGTTGTTAT
IL-10	NM_010548	CCAAGCCTTATCGGAAATGA	TTTTACAGGGGAGAAATCG
Amphiregulin	NM_009704	GACTCACAGCGAGGATGACA	GGCTTGGCAATGATTCAACT
Foxp3	NM_054039	CACCCAGGAAAGACAGCAACC	GCAAGAGCTCTTGTCCATTGA
RORγt	NM_011281	GCGGAGCAGACACACTTACA	TTGGCAAACCTCCACCACATA
STAT3	NM_213659	GAAGACCAAGTTCATCTGTGTG	GTAGCACACTCCGAGGTCAGAT
STAT4	NM_011487	CATCCCTGAAAACCCTCTGA	GACATGGGGAGAAGGTCTGA
STAT6	NM_009284	TGGCCACCATCAGACAAATA	ACCAGGACCATTGACAGGAG



## 2.18 STATISTICAL ANALYSIS

Statistical analysis was performed using the GraphPad InStat software program (GraphPad Prism version 5.0). Differences in the tumour protection effect among various groups were evaluated by one-way ANOVA and Tukey test for multiple comparisons. Dual comparisons were made using the paired Student's *t* test, and  $p < 0.05$  was considered statistically significant. Differences in the survival of mice were analysed using the Kaplan-Meier method, and groups were compared using the Log-rank (Mantel-Cox) test.

## CHAPTER 3: MECHANISTICAL STUDY OF THE EFFECTS OF THE *E. coli*-LLO VACCINE

### 3.1 INTRODUCTION

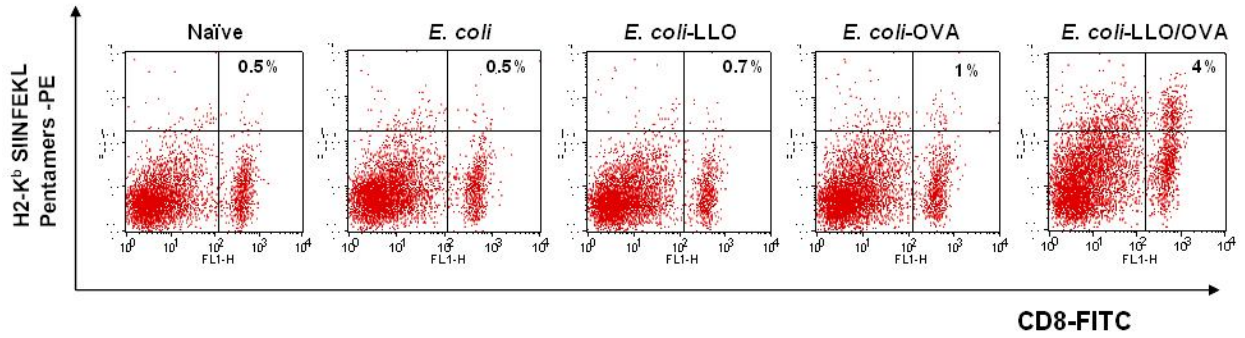
Previous *in-vitro* experiments demonstrated that subcutaneous injection of *E. coli*-LLO expressing OVA antigen (*E. coli*-LLO/OVA) could trigger a very strong anti-tumour response against the highly aggressive B16-OVA melanoma cell line (Radford, Higgins et al. 2002), far superior to that of vaccination using *E. coli* expressing OVA only (*E. coli*-OVA). Considering that improved MHC class I presentation of antigenic peptides is unlikely to be the sole mechanism responsible for the striking difference in efficacy between *E. coli*-OVA or *E. coli*-LLO/OVA vaccines, I wanted to see if CD8<sup>+</sup> T cells are indispensable in tumour eradication. It is crucial to determine how the additional LLO co-expressed within the bacteria can help a marginally-active vaccine (*E. coli*-OVA) to turn into a potent anti-tumour one (*E. coli*-LLO/OVA).

In the study by Gunn GR et al (Gunn, Zubair et al. 2001) with the LLO-antigen (HPV-16 E7 protein) fusion protein secreted by *L. monocytogenes* (*Lm*-LLO-E7), the immunotherapeutic effect could be abrogated by transfer of CD4<sup>+</sup> cells from *Lm*-E7-immunised mice. In addition, the *in-vivo* tumour protection effect was dramatically increased upon CD4<sup>+</sup> or CD25<sup>+</sup> depletion. Regulatory T cells (Treg), usually defined by the expression of CD4 and CD25 in natural Treg, seem particularly to be particularly adept at controlling immune responses to self antigens as well as pathogens. Therefore, I also aimed to determine the impact of LLO-based bacterial vaccine on immune regulation system and the effect of CD25 depletion.

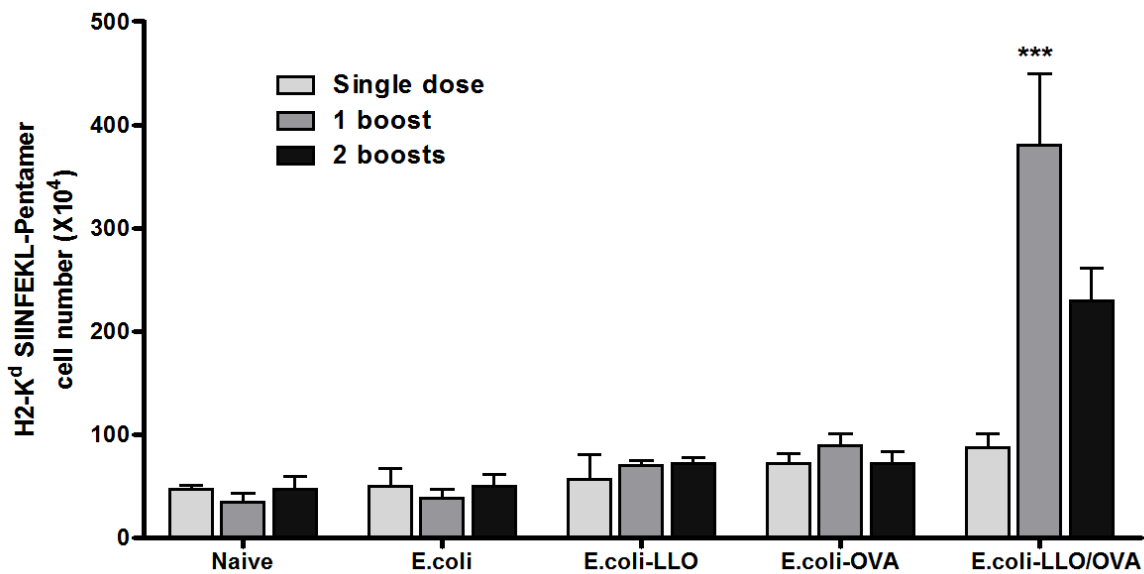
## 3.2 RESULTS

### 3.2.1 H2-K<sup>d</sup>-Pentamer Analysis

To determine the number of OVA specific CTLs, a commercially-available pentamer was used. Animals were given two or three s.c. injections of  $10^8$  paraformaldehyde-fixed *E. coli*, *E. coli*-LLO, *E. coli*-OVA, or *E. coli*-LLO/OVA or PBS, and enumeration of OVA-specific CD8<sup>+</sup> T lymphocytes was performed by FACS 1 week after the last injection. Plots depicting percentages of CD8<sup>+</sup>, pentamer-positive cells gated on live lymphocytes are shown in Figure 4. Mice receiving *E. coli*-OVA vaccines showed a small, but non-significant increase of SIINFEKL/H2-K<sup>b</sup> specific CD8<sup>+</sup> T lymphocytes above the background levels observed in *E. coli*, *E. coli*-LLO, or PBS-vaccinated animals ( $1\pm 0.2$  vs  $0.5\pm 0.2\%$  of total splenocytes, respectively). By contrast, *E. coli*-LLO/OVA-vaccinated mice induced significant levels of SIINFEKL/H2-K<sup>b</sup>-specific CD8<sup>+</sup> T cells. The highest percentage and absolute numbers (4% of total splenocytes,  $3.8\pm 1.2 \times 10^6$  cells) was achieved following one boost injection of *E. coli*-LLO/OVA (Figures 4 and 5). As it has been shown that LLO induces apoptosis of infected cells and activated lymphocytes (Carrero, Calderon et al. 2006), it is likely that multiple boosts may lead to the death of these cells, altering the response. Thus, vaccines combining LLO and OVA allow the activation of OVA-specific CD8<sup>+</sup> T cells, confirming the previously-reported (Radford, Higgins et al. 2002) importance of the incorporation of LLO in the bacterial vaccine.



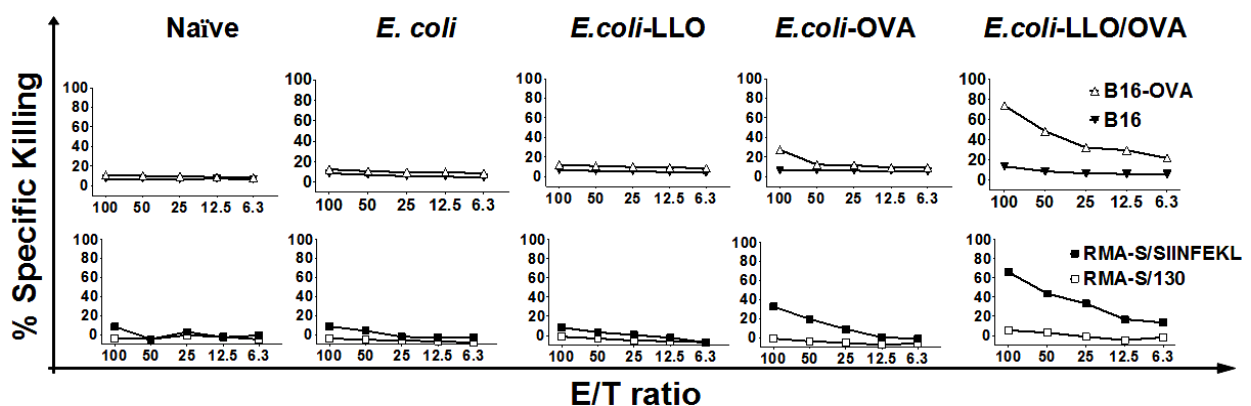
**Figure 4. *E. coli*-LLO/OVA vaccination allows the activation of OVA-specific CD8<sup>+</sup>T cells as measured by multimeric MHC-peptide analyses.** Mice received two immunisations of bacteria at a 1-week interval. Splenocytes were collected 7 days after the last injection and stained with the CD8-FITC and SIINFEKL/H-2K<sup>b</sup> pentamers-PE (ProImmune Ltd, UK). Representative experiments depicting percentages of CD8<sup>+</sup>, pentamer-positive cells gated on live lymphocytes are shown.



**Figure 5. The absolute numbers of SIINFEKL/H-2K<sup>b</sup>-specific T cells in the spleen.** Mice received several immunisations of bacteria at 1-week intervals. Splenocytes were collected 7 days after the last injection and stained with the CD8-FITC and SIINFEKL/H-2K<sup>b</sup> pentamers-PE (ProImmune Ltd, UK) on the live cells (50,000 events) to determine the percentage. The absolute numbers of CD8-Pentamer cells in each mouse were obtained from total splenocyte numbers multiplied by the percentage determined by FACS (mean ± SD); one or two boost injections are shown. \*\*\*  $p < 0.001$ .

### 3.2.2 Specific cytotoxicity

Because OVA-specific CD8<sup>+</sup> T cells expressing the immunodominant T cell epitope OVA<sub>257-264</sub> SIINFEKL are clearly critical to the anti-tumour effect leading to the *in-vivo* rejection of B16-OVA tumour (Bellone, Cantarella et al. 2000; Radford, Higgins et al. 2002), the presence of CTLs specific for SIINFEKL was tested in mice in a prophylactic vaccination setting. To characterise the specificity of the cytotoxic responses, splenocytes from vaccinated mice were re-stimulated *in vitro* with SIINFEKL-loaded LPS-activated splenocytes and specific cytotoxicity was measured 6 days later using SIINFEKL-loaded RMA-S and B16-OVA cells as targets. The results are summarised in Figure 6. Splenocytes from *E. coli*-LLO/OVA-vaccinated animals showed a strong response against SIINFEKL-loaded RMA-S cells and B16-OVA tumour cells (>75% killing at the highest effector to target ratio), suggesting that the CTLs are OVA-specific. By contrast, relatively weak CTL responses against SIINFEKL-loaded RMA-S cells and B16-OVA tumours (< 25% killing at the highest effector to target ratio) were detected in splenocyte cultures from *E. coli*-OVA vaccinated mice, and no cytotoxic activity was found in splenocyte cultures from *E. coli* or *E. coli*-LLO control mice.



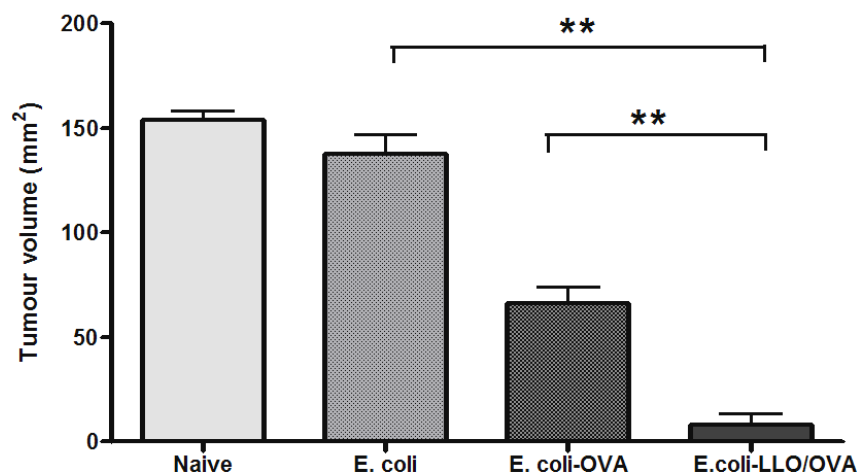
**Figure 6.** *E. coli*-LLO/OVA vaccination generated specific CTL responses. C57BL/6 mice received two s.c. injections of bacteria at a 1-week interval. Splenocytes prepared from

spleens collected 1 week later were restimulated *in vitro* with LPS-stimulated spleen cells loaded with the OVA-restricted class I SIINFEKL peptide. Cytotoxicity was measured 6 days later by <sup>51</sup>Cr-release assay against SIINFEKL (■) or irrelevant peptide-loaded RMA-S (□) cells as well as B16-OVA (△) and the parental B16 tumour cell lines (▼). Results from individual mice are plotted against the E:T ratio, and are representative of two experiments.

### 3.2.3 Tumour Protection Effect

#### 3.2.3.1 Subcutaneous tumour implantation

The next step was to determine whether the specific CTLs observed *in vitro* would provide anti-tumour activity. Mice were first immunised with different bacteria (*E. coli*, *E. coli*-OVA, *E. coli*-LLO/OVA), twice with one week intervals and challenged with B16-OVA cells one week after the last bacterial injection. Figure 7 shows that *E. coli*-LLO/OVA caused a significant tumour inhibition compared to the other groups.



**Figure 7. *E. coli*-LLO/OVA vaccine inhibits subcutaneous tumour growth.** C57BL/6 mice were given two s.c. injections of bacteria at a 1-week interval, then were challenged a week after the boost injection by s.c. implantation of 10<sup>6</sup> B16-OVA cells. Tumour volume was assessed 3 weeks after tumour challenge by the maximal 2-dimension measurement and expressed in mm<sup>2</sup>. Results are expressed as mean ± SD. \*\* *p*<0.01

### 3.2.3.2 Mimicking metastasis

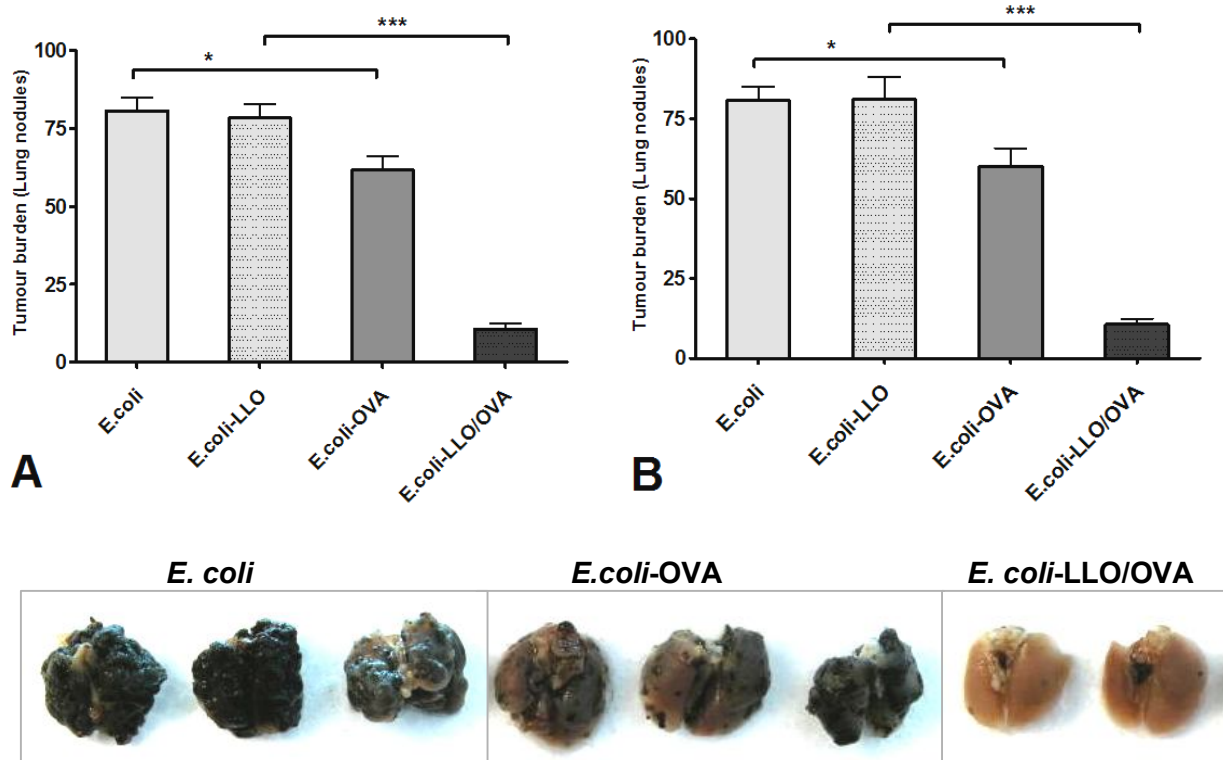
To examine the ability of the vaccines to control the tumour in a metastasis-mimicking model, animals received two injections of each of the *E. coli* vaccines at one week intervals followed by intravenous challenge of  $5 \times 10^5$  B16-OVA cells. In this model, B16-OVA cells target to the lungs where the cells form multiple tumour nodules in lung parenchyma. When the first mice started to show signs of sickness (typically 24-26 days following B16-OVA cells injection), the whole cohort was culled and the tumour-load was accessed by counting tumour nodules in the lungs (100 nodules as maximum).

Figure 8 shows that the lungs of animals vaccinated with *E. coli* or *E. coli*-LLO were intensely infiltrated by metastatic tumours ( $82 \pm 11$  and  $86 \pm 12$  nodules, respectively). The burden was modestly but significantly reduced in mice vaccinated with *E. coli*-OVA ( $60 \pm 12$  per set of lungs,  $p = 0.01$  vs *E. coli*) and dramatically reduced in *E. coli*-LLO/OVA-vaccinated animals ( $5 \pm 2$ ,  $p < 0.0001$  vs. *E. coli*-LLO).

The efficacy of the recombinant *E. coli* vaccines on tumour development was next assessed in a therapeutic setting. Mice challenged with  $5 \times 10^5$  B16-OVA cells were treated with s.c. injections of the various bacteria 8 and 15 days later. As for the vaccination protocol, treatment with *E. coli* or *E. coli*-LLO did not affect the tumour load compared to PBS-vaccinated animals, treatment with *E. coli*-OVA led to a modest but statistically significant reduction in the tumour burden and treatment with *E. coli*-LLO/OVA dramatically reduced the tumour load (Figure 8).

Importantly, none of these effects were observed upon challenge with parental B16 cells (which had been previously performed by Dr. Nitcheu-Tefit), demonstrating

that *E. coli*-LLO/OVA vaccine resulted in the establishment of an appropriate adaptive OVA-specific immune response.

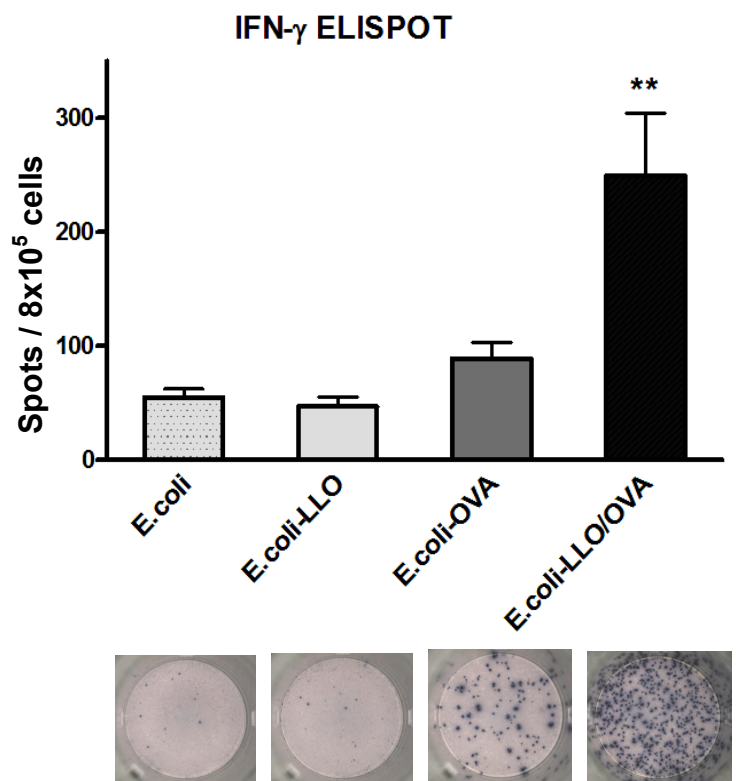


**Figure 8. *E. coli*-LLO/OVA vaccine inhibits lungs metastasis induced by tail vein injection of B16-OVA cells.** (A) C57BL/6 mice were given two s.c. injections of bacteria at a 1-week interval, then were challenged a week after the boost injection by tail-vein injection of  $5 \times 10^5$  B16/OVA cells. Animals were sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumour challenge) and the tumour growth was assessed by counting tumour nodules in the lungs. (B) C57BL/6 mice were first injected by tail-vein injection of  $5 \times 10^5$  B16/OVA cells and given two s.c. injections of bacteria on Day 8 and 15. Animals were sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumour challenge) and the tumour growth was assessed by counting tumour nodules in the lungs. Results are expressed as mean  $\pm$  SD. The experiments presented are representative of at least two experiments involving six animals per group. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ . (Lower panel) Excised lung specimens were obtained from *E. coli*-, *E. coli*-OVA-, and *E. coli*-LLO/OVA-vaccinated mice, which clearly demonstrated the effects of tumour metastasis by *E. coli*-LLO/OVA vaccine.



### 3.2.4 CD8-mediated Cytokine Responses upon Bacterial Vaccination

The cytokine responses to different bacterial vaccines in the sera were analysed by ELISpot and ELISA. The IFN- $\gamma$  ELISpot demonstrated that *E. coli*-LLO/OVA vaccination could largely enhance CD8-mediated responses compared to *E. coli*, *E. coli*-LLO, and *E. coli*-OVA (Figure 9). ( $250 \pm 84$  spots/well vs  $48 \pm 8$  or  $80 \pm 16$  spots/well in *E. coli* and *E. coli*-OVA vaccinated animals, respectively;  $p = 0.004$ ).



**Figure 9. IFN- $\gamma$  ELISpot responses in immunised mice.** Mice received two s.c. injections of bacteria at a 1-week interval. Splenocytes were collected after the boost injection and incubated overnight with the SIINFEKL peptide, and ELISpot assay was used to measure IFN- $\gamma$  secretion. Results are expressed as mean  $\pm$  SD. Splenocytes from mice that received vaccines coexpressing LLO and OVA induced a significant strong response (\*\*,  $p = 0.0043$ ). The experiments presented are representative of two separate experiments involving three animals per group. (Lower panel) Representative plates from ELISpot assay.

Sera were collected one week after the boost vaccination, in SIINFEKL-stimulated splenocytes cultures (from spleens collected one week after the boost vaccination), and in BMDCs cultures activated by the various *E. coli*, as described in *materials and methods*. Results are summarised in Table 5. IL-2, IL-4, IL-10 and IFN- $\gamma$  serum levels, analysed by ELISA, were just above the threshold of detection and not significantly different between any of the animals given the *E. coli* vaccines. IFN- $\gamma$  levels were significantly lower in splenocyte cultures from animals that received the *E. coli*-OVA vaccines as compared to animals that received *E. coli*-LLO/OVA vaccines ( $450\pm 14$  vs.  $841\pm 102$  pg/ml). The same trend was found in BMDCs cultures that were activated by *E. coli* and *E. coli*-OVA as compare to BMDCs cultures that were activated by *E. coli*-LLO and *E. coli*-LLO/OVA, while the inverse correlation was observed for IL-10 production. Altogether, these data indicate that *E. coli*-LLO/OVA vaccine is more potent in stimulating CD8-mediated cytokine responses.

	IL-4	IL-10	IFN- $\gamma$	IL-2
<b>Serum levels (pg/ml)<sup>a</sup></b>				
Naive	0.8	7.9	4.7 (0–9.4)	7.6 (0–15.6)
<i>E. coli</i>	0.7 (0–3.5)	9 (2.8–24.8)	5 (0.5–9.7)	24 (0–45)
<i>E. coli</i> LLO	0	7.5 (3.3–10.8)	14 (6.4–19.7)	12.8 (0–40)
<i>E. coli</i> OVA	11 (0–36)	4.5 (3.3–5.4)	11.5 (5.9–13)	11 (0–17)
<i>E. coli</i> LLO/OVA	0	15.3 (4.2–34.8)	3.9 (0–9.7)	15.2 (0–44)
<b>BMDCs cultures (pg/ml)<sup>b</sup></b>				
Naive	7.9 $\pm$ 0.5	10 $\pm$ 3.5	5 $\pm$ 2	14 $\pm$ 5
<i>E. coli</i>	6.2 $\pm$ 1.1	17 $\pm$ 13	7 $\pm$ 1.3	15 $\pm$ 2.2
<i>E. coli</i> LLO	6.2 $\pm$ 0.9	25 $\pm$ 12	20 $\pm$ 6.2	17 $\pm$ 2.5
<i>E. coli</i> OVA	6.4 $\pm$ 0.25	140 $\pm$ 21	5 $\pm$ 1.1	15 $\pm$ 6.5
<i>E. coli</i> LLO/OVA	6.5 $\pm$ 1.3	44 $\pm$ 12	40 $\pm$ 9***	16 $\pm$ 5.3
	IL-4		IL-10	
	Unstimulated	Stimulated	Unstimulated	Stimulated
<b>Splenocyte cultures (pg/ml)<sup>c</sup></b>				
Naive	12 $\pm$ 2.2	9.2 $\pm$ 2.1	11.2 $\pm$ 2.6	9.6 $\pm$ 4.2
<i>E. coli</i>	9.2 $\pm$ 3.1	9.1 $\pm$ 3.5	8.8 $\pm$ 5.4	12.3 $\pm$ 4.1
<i>E. coli</i> LLO	7.5 $\pm$ 0.5	8.5 $\pm$ 0.1	8.8 $\pm$ 1.2	9.8 $\pm$ 2.5
<i>E. coli</i> OVA	8 $\pm$ 2.5	9.6 $\pm$ 2.3	10.6 $\pm$ 3.3	10.8 $\pm$ 4.2
<i>E. coli</i> LLO/OVA	9.8 $\pm$ 6.3	12.5 $\pm$ 1.2	8 $\pm$ 2.3	11 $\pm$ 3.5
	IFN- $\gamma$		IL-2	
	Unstimulated	Stimulated	Unstimulated	Stimulated
<b>Splenocyte cultures (pg/ml)<sup>c</sup></b>				
Naive	152.3 $\pm$ 13.2	172.3 $\pm$ 9.6	30.2 $\pm$ 11	31.9 $\pm$ 13
<i>E. coli</i>	208 $\pm$ 22.3	332 $\pm$ 30	28.3 $\pm$ 6.5	20.4 $\pm$ 10.3
<i>E. coli</i> LLO	140 $\pm$ 46.4	170 $\pm$ 14	23.9 $\pm$ 5.9	20.2 $\pm$ 3.6
<i>E. coli</i> OVA	176 $\pm$ 37	450 $\pm$ 14.2	22.2 $\pm$ 3.2	26.7 $\pm$ 6.2
<i>E. coli</i> LLO/OVA	126 $\pm$ 52	841 $\pm$ 102***	21 $\pm$ 8.7	25.7 $\pm$ 1.4

**Table 5. CD8-mediated cytokine responses in the sera of immunised animals.**

<sup>a</sup> Mice received two vaccinations of  $10^8$  *E. coli*, *E. coli*-LLO, *E. coli*-OVA, or *E. coli*-LLO/OVA at a 1-week interval. Sera were collected 1 week after the boost injection and were analysed for cytokine production by ELISA. The experiments presented are representative of two separate experiments involving six animals per group.

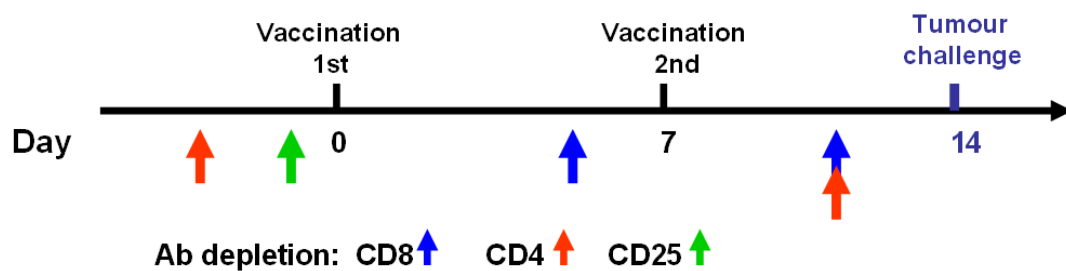
<sup>b</sup> BMDCs were pre-activated with different bacteria as described in *Materials and Methods* then cultured for 48 h before being assayed for cytokine production. The experiments presented are representative of two separate experiments. \*\*\*,  $p < 0.001$ , *E. coli*-OVA vs. *E. coli*-LLO/OVA.

<sup>c</sup> Mice received two injections of  $10^8$  *E. coli*, *E. coli*-LLO, *E. coli*-OVA, or *E. coli*-LLO/OVA at a 1-week interval. Single-cell suspensions were prepared from the spleens collected 1 week after the boost injection. Splenocytes were left unstimulated or were restimulated *in vitro* for 3 days with the SIINFELK peptide before being assayed for cytokine production. The experiments presented are representative of two separate experiments involving three animals per group. \*\*\*,  $p < 0.001$  *E. coli*-OVA vs. *E. coli*-LLO/OVA.

### 3.2.5 Antibody Depletion Study

Depletion of CD4<sup>+</sup> or CD25<sup>+</sup> T cells in *E. coli*-OVA vaccinated animals allows the establishment of CD8<sup>+</sup> T cell-mediated tumour-protective immune responses

To assess the relative contribution of T cell subsets in tumour protection, CD8<sup>+</sup> as well as CD4<sup>+</sup> and CD25<sup>+</sup> cells were depleted *in vivo* (Figure 10) in the vaccination model. CD4 depletion was carried out 5 days before and 10 days after the first bacterial injection by intraperitoneal injections of the GK1.5 mAb. CD8 depletion experiments were conducted using intraperitoneal injections of the YTS169.4 mAb (on days 5 and 10 after the first vaccination).



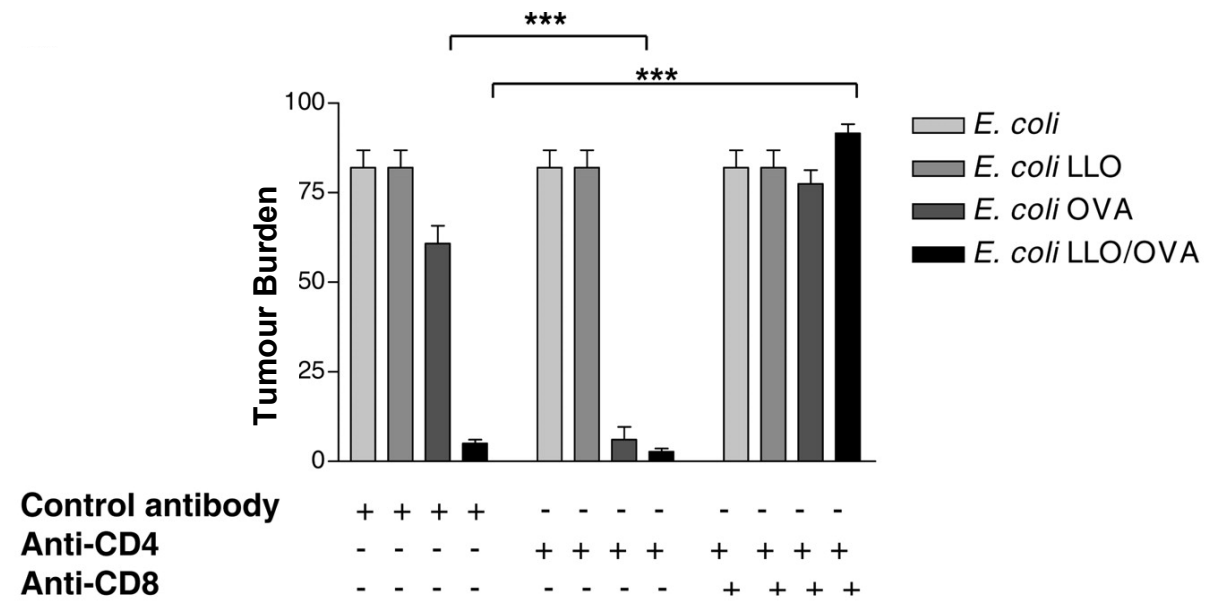
**Figure 10.** Schematic representation of the specific Ab depletion in bacterial immunisation. CD4 or CD25 depletion *in vivo* was started before the first immunisation (at the T-cell priming stage) and CD8 depletion was performed after the first immunisation (at the effector stage).

CD8 depletion abolished the protection in *E. coli*-LLO/OVA vaccinated animals as well as the modest but significant protection observed upon *E. coli*-OVA vaccination, demonstrating the indispensable role of CD8<sup>+</sup> T cells in tumour protection (Figure 11). Depletion of CD4<sup>+</sup> T cells at the stage of T cell priming had no impact on *E. coli*-LLO/OVA vaccination, suggesting that CD8<sup>+</sup> T cell priming *in vivo*

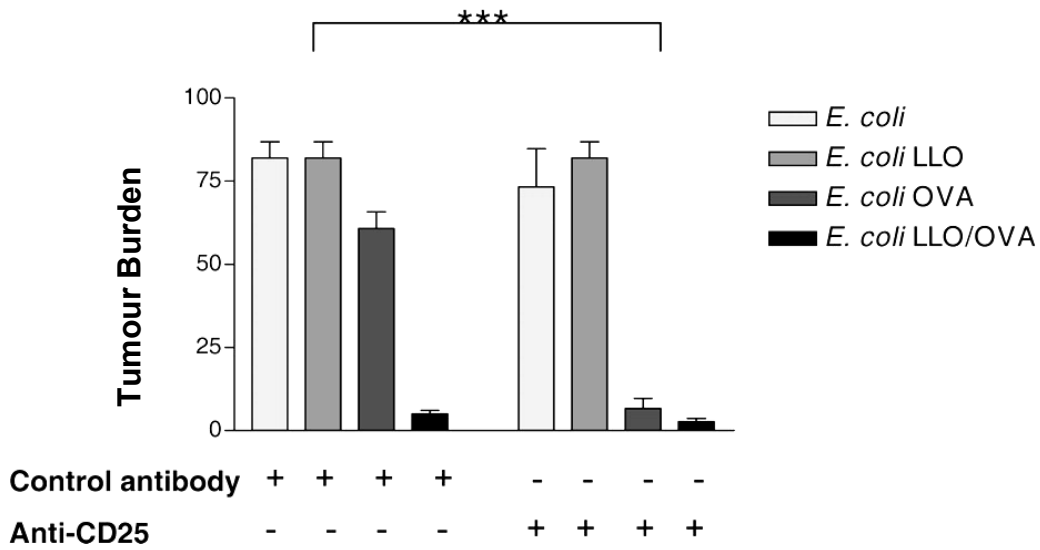
can occur in the absence of CD4<sup>+</sup> T cell help. However, CD4 depletion unexpectedly turned *E. coli*-OVA into a vaccine as effective as *E. coli*-LLO/OVA suggesting that a subset of CD4<sup>+</sup> cells inhibited the cells mediating the anti-tumour response. Moreover, mice receiving combined treatments with depleting antibodies against CD4 and CD8 showed progressive tumour growth, confirming that the anti-tumour activity unmasked by CD4<sup>+</sup> T cell depletion is dependent on the presence of CD8<sup>+</sup> T cells (Figure 11).

I next attempted to characterise the CD4 subset inhibiting the CD8 response. NK cells depleted with PK136 antibodies allowed us to exclude the possibility of any regulation by NK or NKT cells (Dr. J Nitcheu-Tefit). CD4<sup>+</sup>CD25<sup>high</sup> Treg have been shown to enhance tumour growth by regulating cells mediating tumour immunosurveillance (Sakaguchi 2004). Treg may therefore exert suppressor functions following *E. coli*-OVA vaccination. To assess the involvement of these cells, mice were depleted of CD25 cells by i.p. administration of the PC61 mAb (anti-CD25) one day before bacterial vaccination and the tumours were inoculated one week after the boost vaccination. The tumour burden in different conditions is presented in Figure 11. When compared to depletion using an irrelevant control mAb (RatIGg1), CD25 depletion had no statistically significant effect on the number of tumour nodules in mice vaccinated either with *E. coli* or *E. coli*-LLO/OVA. By contrast, this depletion resulted in a dramatic, statistically significant reduction in the tumour load in *E. coli*-OVA-vaccinated animals ( $p < 0.001$  compare to control Ab-treated mice), turning *E. coli*-OVA into a vaccine as potent as *E. coli*-LLO/OVA. Interestingly, IFN- $\gamma$  secretion, as measured by ELISpot analysis, was restored in mice that received *E. coli*-OVA vaccines and that were depleted of CD4<sup>+</sup> or CD25<sup>+</sup> cells (Figure 13). Splenocytes from Ab-treated controls stimulated low IFN- $\gamma$  secretion ( $50 \pm 16$  spots/well) that was

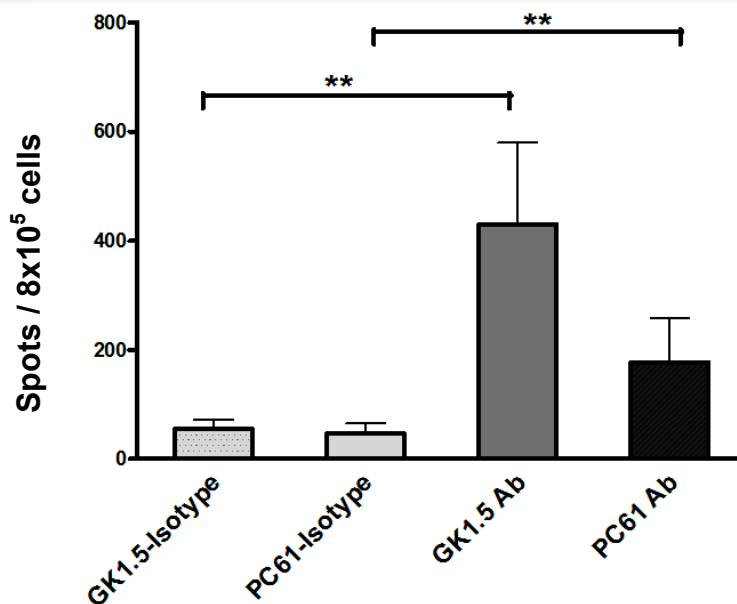
boosted by CD4 depletion ( $400 \pm 250$  spots/well,  $p = 0.002$ ) or by CD25 depletion ( $175 \pm 90$  spots/well,  $p = 0.001$ ) (Figure 13). These data strongly suggest that *E. coli*-OVA vaccines induce OVA-specific CD8<sup>+</sup> T cells that are necessary for the anti-tumour effect but that Treg cells prevent their expansion and effector function.



**Figure 11. CD8<sup>+</sup> T cells are the key mediators for tumour protection and CD4<sup>+</sup> cell depletion enhanced anti-tumour effect in mice receiving the *E. coli*-OVA vaccines. a.** Mice received two s.c. injections of bacteria on days 0 and 7. Depletions were conducted by i.p. injection of GK1.5-depleting mAb (anti CD4) at the stage of T cell priming (on days -5 and 10) in combination or not with i.p. injections of YTS169.4 (anti-CD8) on days 10 and 17. As a control, an irrelevant mAb control (PYLT-1) was used. Mice were challenged on day 14 by tail-vein injection of  $5 \times 10^5$  B16-OVA cells and sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumour challenge), then the tumour load was assessed in the lungs. Results are expressed as mean  $\pm$  SD. The experiments presented are representative of two experiments involving six animals per groups; \*\*\*,  $p < 0.001$ .



**Figure 12. CD25<sup>+</sup> cells depletion effects.** Depletion of CD25<sup>+</sup> cells was conducted by i.p. injection of the PC61 mAb on day -1 before s.c. bacterial vaccinations on days 0 and 7. As a control, an irrelevant mAb (RatIGg1) was used. Mice were challenged on day 14 by tail-vein injection of  $5 \times 10^5$  B16/OVA cells and sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumour challenge), then the tumour load was assessed in the lungs. Results are expressed as mean  $\pm$  SD. The experiments presented are representative of two experiments involving six animals per groups. \*\*\*,  $p < 0.001$ .



**Figure 13. IFN- $\gamma$  ELISpot responses following CD4<sup>+</sup>/CD25<sup>+</sup> depletion.** Depletion of CD4<sup>+</sup> or CD25<sup>+</sup> cells was conducted as previously described, then splenocytes, from *E. coli*-OVA-immunised mice, harvested 1 week after the boost injection were incubated overnight with the SIINFEKL peptide. ELISpot assay was used to measure INF- $\gamma$  secretion. The experiments presented are representative of three experiments involving three animals per

groups. Results are expressed as mean  $\pm$  SD. \*\*,  $p = 0.0016$  (GK1.5 vs isotype Ab control treatment); \*\*,  $p = 0.0023$  (PC61 vs isotype control treatment).

### 3.2.6 Analysis of Treg Prevalence and Phenotype

In the experience of Gunn et al, the *Listerial* vaccine altered Treg prevalence and relocation to tumour microenvironment (Gunn, Zubair et al. 2001). To investigate whether LLO expression affects Treg expansion, we compared the prevalence of these cells in the spleen and draining lymph nodes close to the site of inoculation in vaccinated animals. No significantly different CD4<sup>+</sup>CD25<sup>high</sup> T cell allocation was found within the spleens and inguinal lymph nodes in all groups of mice, as assessed by FACS analysis, and Foxp3<sup>+</sup> expression within this population was similar (80-100%) (Table 6)

To further investigate whether LLO expression in the bacterial vaccine could affect the expansion of Treg, splenocytes from naïve mice were cultured with the various *E. coli*-activated BMDCs, used as APCs, and the kinetics of CD4<sup>+</sup>CD25<sup>high</sup> Treg appearance were established in the cultures. The level of expression of co-stimulatory and MHC class I/II molecules on BMDCs were similar following activation with the various bacteria (Table 7).

	Spleens		Inguinal LNs	
	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 (%)
Naive	3.66 $\pm$ 0.66	96 $\pm$ 0.86	3.24 $\pm$ 0.5	89.57 $\pm$ 2.1
<i>E. coli</i>	4.13 $\pm$ 0.52	96.94 $\pm$ 2.6	3.26 $\pm$ 0.06	82.34 $\pm$ 2.7
<i>E. coli</i> LLO	4.17 $\pm$ 0.82	91.33 $\pm$ 2.4	3.89 $\pm$ 0.27	88.75 $\pm$ 2.06
<i>E. coli</i> OVA	3.46 $\pm$ 0.54	93.56 $\pm$ 3.7	3.37 $\pm$ 1	86.11 $\pm$ 8.6
<i>E. coli</i> LLO/OVA	4.18 $\pm$ 0.15	93.9 $\pm$ 6.7	3.12 $\pm$ 1.13	73.88 $\pm$ 9.5

**Table 6. Treg prevalence in spleens and drainage LNs.** Single-cell suspensions were prepared from the spleens and inguinal LNs collected 7 days after the boost injection then stained with the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cell Staining kit (eBioscience). CD4<sup>+</sup>CD25<sup>high</sup> T cell frequencies and Foxp3 expression within this population as measured

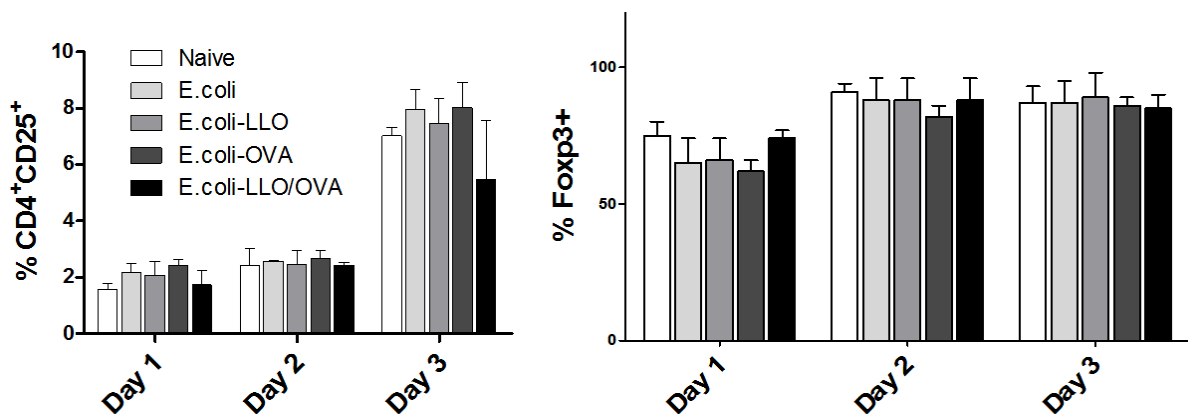


by FACS analysis are shown. The data presented are representative of two separate experiments involving six animals per group.

	CD40	CD80	CD86	IA <sup>b</sup>	H2-K <sup>b</sup>
<i>E.coli</i> -LLO/OVA primed BMDC	69.52	84.08	74.10	84.80	78.58
<i>E.coli</i> -OVA primed BMDC	69.58	83.39	76.45	84.61	78.21

**Table 7. Expression of costimulation molecules of activated BMDCs.** BMDCs incubated with either *E. coli*-LLO/OVA or *E. coli*-OVA for 24h and the expression of surface molecules, CD40, CD80, CD86, and MHC class I and MHC class II complexes were analysed by FACS.

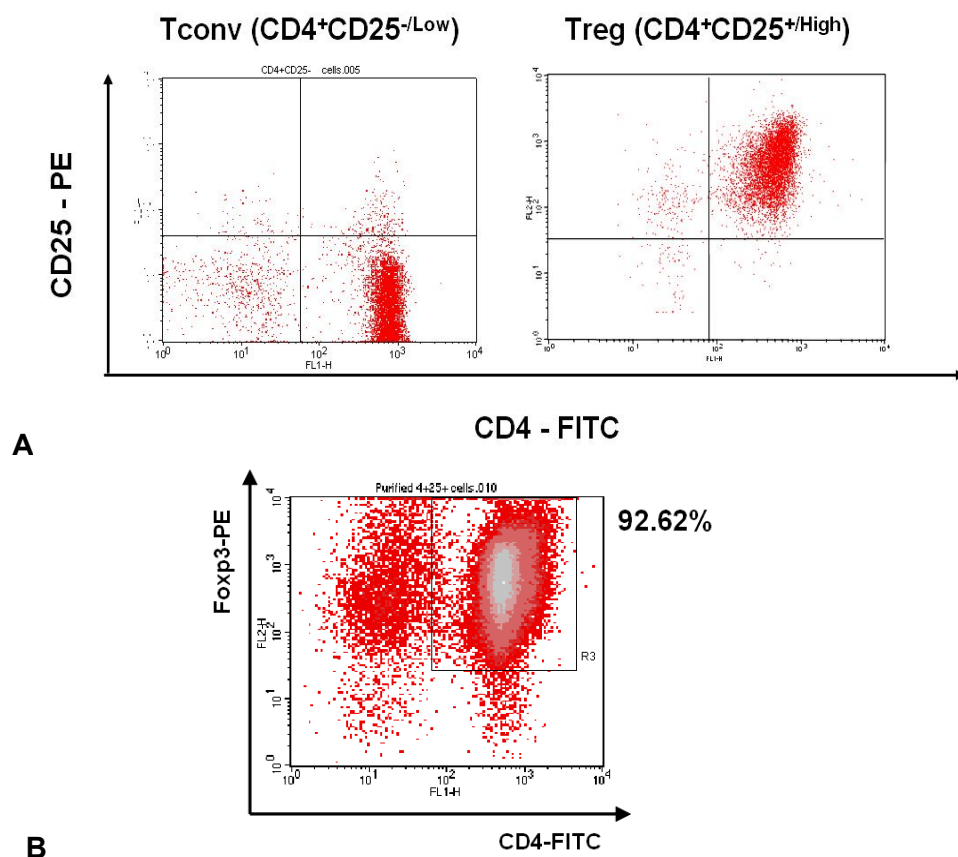
CD4<sup>+</sup>CD25<sup>high</sup> T cells arising from the cultures increased over time, however no significant differences in the frequencies as well as in Foxp3 expression were noticeable between groups (Figures 14 and 15). From these results I concluded that LLO expression in the vaccine does not affect antigen-induced Treg expansion.



**Figure 14 and 15. Induced Treg from bacteria-activated BMDCs.** Splenocytes prepared from naive C57BL/6 mice were cultured with BMDCs that have been previously activated with *E. coli*, *E. coli*-LLO, *E. coli*-OVA, or *E. coli*-LLO/OVA. Cells were harvested at different time points then stained for CD4, CD25, and Foxp3 expression (eBioscience Treg Staining kit) before being analysed by FACS. The experiments presented are representative of two separate experiments.

### 3.2.7 Treg Functional Assay

Since the prevalence and Foxp3 expression of Treg in spleens and drainage lymph nodes was not significantly affected by the *E. coli*-LLO vaccine, it was speculated that the immunosuppressive function of Treg had been changed. Therefore, an *in-vitro* assay to assess the functionality of Treg was required to confirm the speculation. CD4<sup>+</sup>CD25<sup>high</sup> cells were separated by magnetic antibody cell sorting (MACS, Miltenyi Biotech, Germany) according to the manufacturer's instructions. The purity of cells was confirmed by FACS analysis, and > 90% of the cells were shown to be either CD25<sup>low</sup> or CD25<sup>high</sup> (Figure 16).

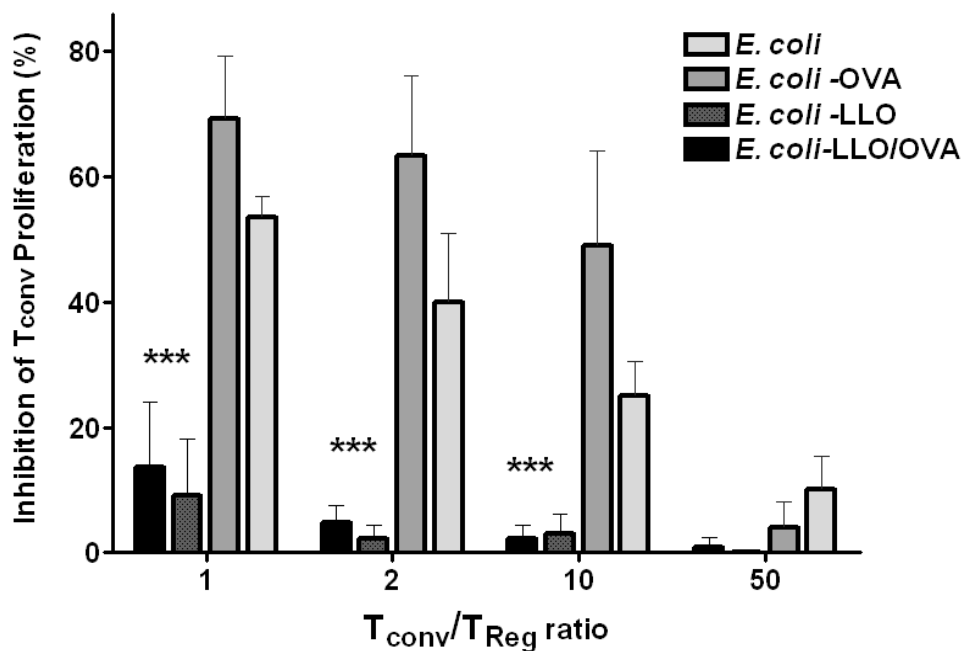


**Figure 16. Purity of *in vitro* sorted Tconv and Treg cells.** (A) Purified CD4<sup>+</sup> cells: Tconv (CD4<sup>+</sup>CD25<sup>low</sup>) and Treg (CD4<sup>+</sup>CD25<sup>high</sup>) subsets, were stained with CD4-FITC and analysed on FACSCalibur (BD Biosciences). Both cell groups showed >90% purity upon

FACS analysis. (B) Most of the purified CD4<sup>+</sup>CD25<sup>high</sup> cells (>90%) positively expressed the master regulator of Treg functions, Foxp3.

*E. coli*-LLO vaccination reduced the Treg suppression on Tconv proliferation

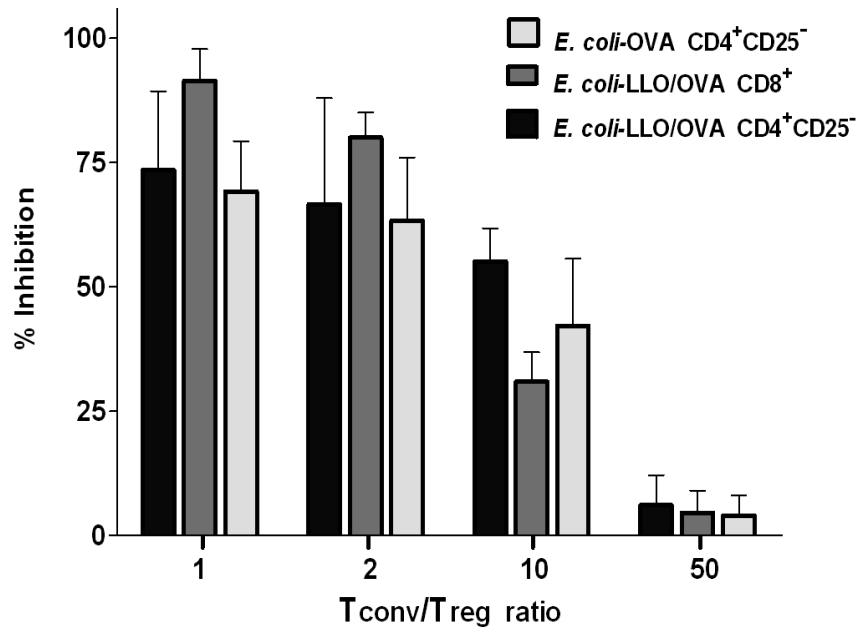
To test their functionality, Treg were purified after the vaccination regimen and yielded a CD4<sup>+</sup>CD25<sup>high</sup> population that was more than 90% pure. I performed titration studies with different amounts of Treg mixed with their corresponding CD4<sup>+</sup>CD25<sup>low</sup> responders (Tconv) (10<sup>5</sup> per assay). Cultures were stimulated with a purified anti-CD3 monoclonal antibody. Treg from each of the vaccine systems did not proliferate upon TCR stimulation (not shown). In the mixed leukocyte reaction, Tconv proliferation from *E. coli* or *E. coli* OVA-vaccinated animals was gradually reduced according to Treg dosing, and > 50% inhibition was observed at a Tconv/Treg cells ratio of 1:1 (Figure 17). By contrast, inhibition of Tconv proliferation was dramatically reduced with Treg cells from *E. coli*-LLO or *E. coli*-LLO/OVA-vaccinated mice (< 20% at the highest ratio).



**Figure 17. Altered Treg suppression by *E. coli*-LLO vaccine.** CD4+CD25+ T cells isolated from *E. coli*-OVA or *E. coli*-LLO/OVA-vaccinated animals induce different levels of suppression. Mice received two s.c. injections of *E. coli*, *E. coli*-LLO, *E. coli*-OVA, or *E. coli*-LLO/OVA and splenocytes prepared from spleens harvested 7 days after the boost injection were separated into CD4+CD25+ and CD4+CD25- cells. In a MLR, CD4+CD25<sup>high</sup> (variable number) were cultured with CD4+CD25<sup>-</sup> (10<sup>5</sup>) T cells for 4 days in the presence of 0.5 µg/ml anti-CD3 and 2 x 10<sup>5</sup> naive irradiated splenocytes. Proliferation of responders was measured by adding <sup>3</sup>H-Thymidine for the last 18 h of culture period and inhibition of proliferation was determined. The experiments presented are representative of three independent experiments. \*\*\*, p < 0.001 *E. coli* vs *E. coli*-LLO and *E. coli*-OVA or *E. coli*-LLO/OVA.

#### *The susceptibility of Tconv was not altered*

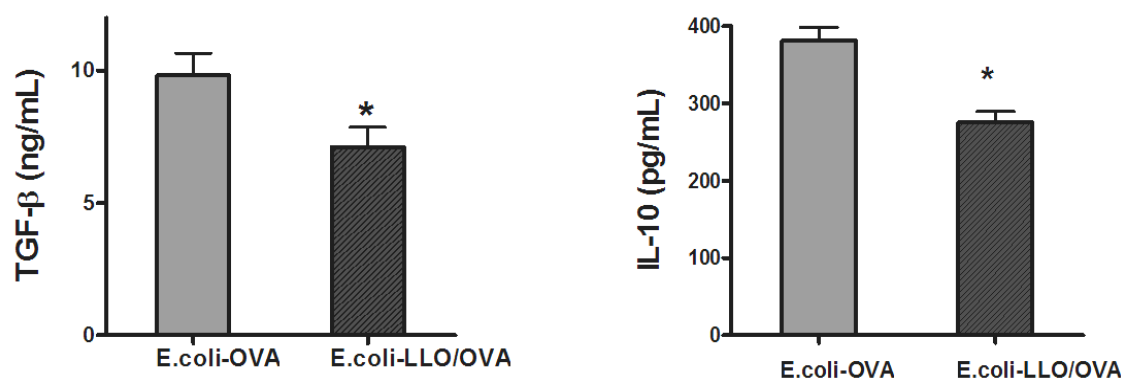
To confirm that the observations were only due to a loss of Treg function, responder cells from *E. coli*-LLO-vaccinated animals were mixed with Treg from *E. coli*-vaccinated animals and vice versa. The question was whether these reduced suppressions were attributable to suppressors or responders. Was the *E. coli*-LLO vaccine prone to activate the Tconv with resisting Treg effects? Therefore, I tested the Tconv proliferation capabilities from different vaccination strategies. Treg from *E. coli*-OVA vaccine system inhibited the proliferation of CD4<sup>+</sup>CD25<sup>low</sup> or CD8<sup>+</sup> responders from *E. coli*-LLO/OVA system as effectively as they inhibited their corresponding responders (Figure 18), showing that the vaccine did not affect the susceptibility of Tconv to Treg suppression. Thus animals receiving *E. coli*-LLO or *E. coli*-LLO/OVA vaccines had reduced overall Treg function compared to Treg from mice receiving *E. coli* or *E. coli*-OVA vaccines, suggesting that LLO expression in the *E. coli* vaccines inhibits Treg function.



**Figure 18. Unaltered Tconv susceptibility to Treg suppression.** Mice received two s.c. injections of *E. coli*-OVA or *E. coli*-LLO/OVA and splenocytes prepared from spleens harvested 7 days after the boost injection were separated into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. CD8<sup>+</sup> T cells were also purified from *E. coli*-LLO/OVA-vaccinated animals. In a MLR, CD4<sup>+</sup>CD25<sup>+</sup> (variable numbers) from *E. coli*-OVA were cultured with 10<sup>5</sup> of their corresponding responders (CD4<sup>+</sup>CD25<sup>-</sup> T cells) or responders from *E. coli*-LLO/OVA-vaccinated animals (CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup> T cells) for 4 days in the presence of 0.5 µg/ml anti-CD3 and 2 x 10<sup>5</sup> naive irradiated splenocytes. Proliferation of responders was measured by adding <sup>3</sup>H-Thymidine for the last 18 h of culture period and inhibition of proliferation was determined.

### 3.2.8 Suppressive Cytokine Profiles in Activated Treg

Suppression by inhibitory cytokines is one of the proposed potential mechanisms related to Treg from a functional perspective. TGF- $\beta$  and IL-10 are the key cytokines within this context. From the sera of vaccinated animals, there is no significant difference in the level of inhibitory cytokines (Table 5). However, the activated Treg from *E. coli*-LLO/OVA secreted higher levels of TGF- $\beta$  and IL-10 than *E. coli*-OVA in response to anti-CD3+IL-2 stimulation in culture (Figures 19 and 20).



**Figures 19 and 20. Suppressive cytokines secreted by the activated Treg.** CD4<sup>+</sup>CD25<sup>+</sup> populations obtained from the spleens of *E. coli*-OVA- and *E. coli*-LLO/OVA- vaccinated mice were stimulated in culture with or with a combination of IL-2 (10 ng/ml) and anti-CD3 Ab (0.5  $\mu$ g/ml) for 5 days. TGF- $\beta$  and IL-10 production were measured by ELISA (\*  $p < 0.05$ ).

### 3.3 CONCLUSION

The results of this project showed that, following vaccination or treatment in mice, *E. coli*-LLO/OVA bacterial vaccine demonstrated remarkable levels of protection against OVA-expressing tumour cells. By contrast, *E. coli*-OVA showed rather poor protection. OVA-specific cytotoxic T lymphocytes were induced in *E. coli*-LLO/OVA vaccinated mice, and CD8<sup>+</sup> depletion, but not NK cell depletion, completely abolished

the anti-tumour activity of the *E. coli*-LLO/OVA vaccine. Similarly to the results observed by Gunn et al (Gunn, Zubair et al. 2001), CD4<sup>+</sup> depletion turned *E. coli*-OVA into a vaccine as effective as *E. coli*-LLO/OVA and recovered the cytokine responses suggesting that a subset of CD4<sup>+</sup> cells suppressed the CD8<sup>+</sup> T cell-mediated anti-tumour response. Subsequent data demonstrated that these suppressive cells consisted of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells. I found that, although CD4<sup>+</sup>CD25<sup>high</sup> expansion and Foxp3 expression within this population was similar in vaccinated animals, Treg cells from *E. coli*-LLO/OVA vaccinated animals were unable to suppress conventional T cell (Tconv) proliferation. These findings provide the first evidence that LLO expression affects Treg cell function and may have important implications for enhancing anti-tumour vaccination strategies in humans.

## **CHAPTER 4: EXPLORING THE T<sub>REG</sub> SUPPRESSION SIGNATURE BY MICROARRAY ANALYSES**

### **4.1 INTRODUCTION**

Treg cells control the delicate balance between immunity and tolerance, explaining their important role in autoimmune diseases, cancer, transplantation tolerance, and even allergy. In the field of immunotherapy, the correlation of treatment efficacy and the impact on Treg has been increasingly discussed. However, only a handful of publications has addressed this issue but none of them has proposed the mechanism of how the inhibitory nature of Treg is affected by the vaccine. From the results of Chapter 3 which show the effect of *E. coli*-LLO vaccine on Treg, I intended to identify the differences in inactive versus functional Treg.

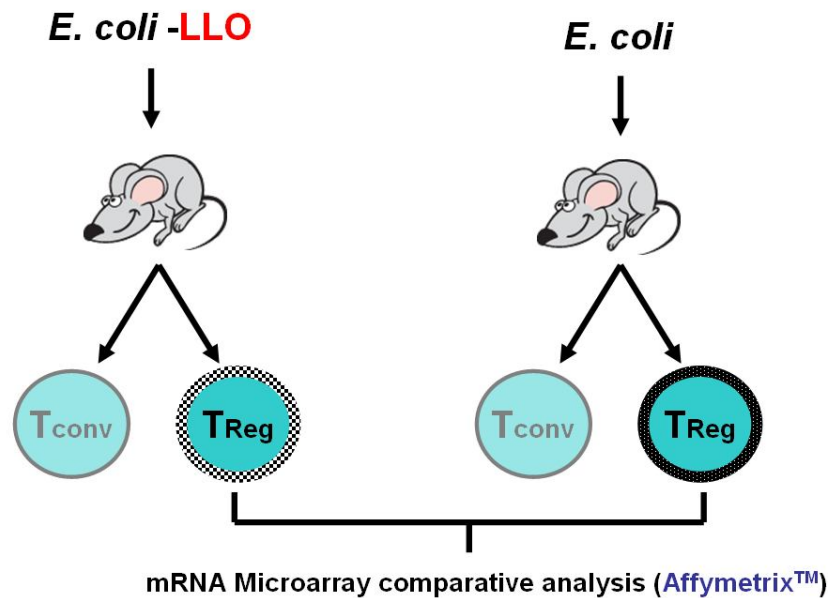
Microarrays have illustrated their potential to unravel gene expression of various subsets of leukocytes. There have been a few studies analysing gene expression in Treg (compared to other T cell groups) using microarrays (Pfoertner, Jeron et al. 2006; Sfanos, Bruno et al. 2008), but none with comparison of differently-conditioned Treg. Our microarray analysis aimed at determining the difference in Treg gene expression under different immunisation conditions.

### **4.2 EXPERIMENTAL SETTING**

Previous experiments in Chapter 3 have demonstrated that LLO-expressing *E. coli* vaccine can dampen Treg suppressive effects through functional reversal instead of reducing Treg prevalence (Nitcheu-Tefit, Dai et al. 2007). A comparative microarray analysis to screen the genome-wide mRNA expression difference between the

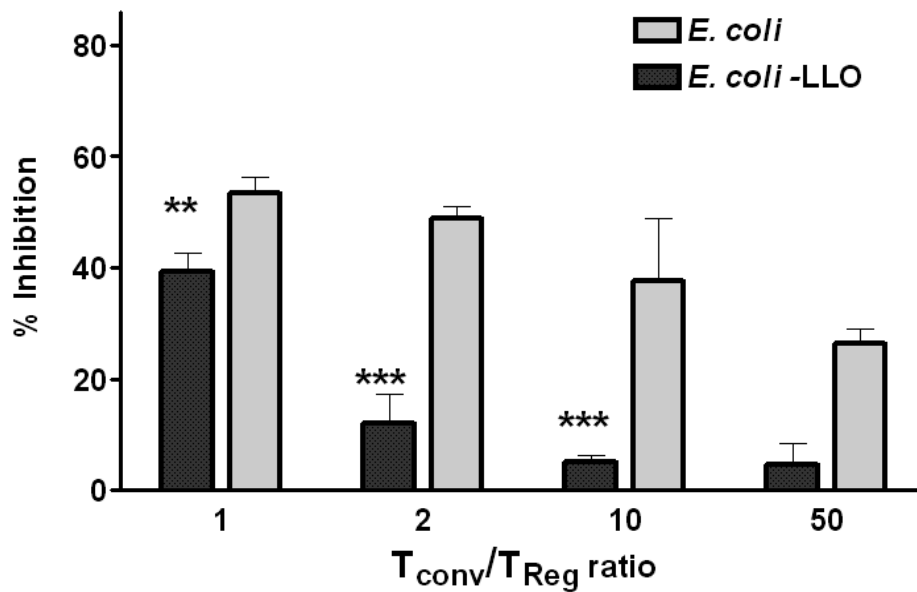


primary Treg from *E. coli*- and *E. coli*-LLO-immunised mice was initiated. Pooled splenocytes were harvested from 10 mice, which had received three bacterial vaccinations with one-week intervals (Figure 21). CD4<sup>+</sup>CD25<sup>High</sup> Treg were purified and total RNA (between 1.0 and 2.0 µg in each group) was extracted by Trizol and Chloroform as described in Chapter 2.



**Figure 21. Schematic representation of the experiment setting for comparative microarray.** 10 mice were immunised with  $10^8$ /mouse *E. coli* or *E. coli*-LLO 3 times at 1-week intervals. Total pooled splenocytes were harvested one week after the last vaccination and Treg/Tconv were separated by MACS (Miltenyi Biotech).

To check the degree of functionality of the two Treg populations, simultaneous mixed Treg/Tconv proliferation assay was carried out when harvesting the splenocytes from immunised mice. The data showed that there is a significant difference of Treg suppression on Tconv proliferation between *E. coli* and *E. coli*-LLO-vaccinated group in various Tconv/Treg ratios (Figure 22).



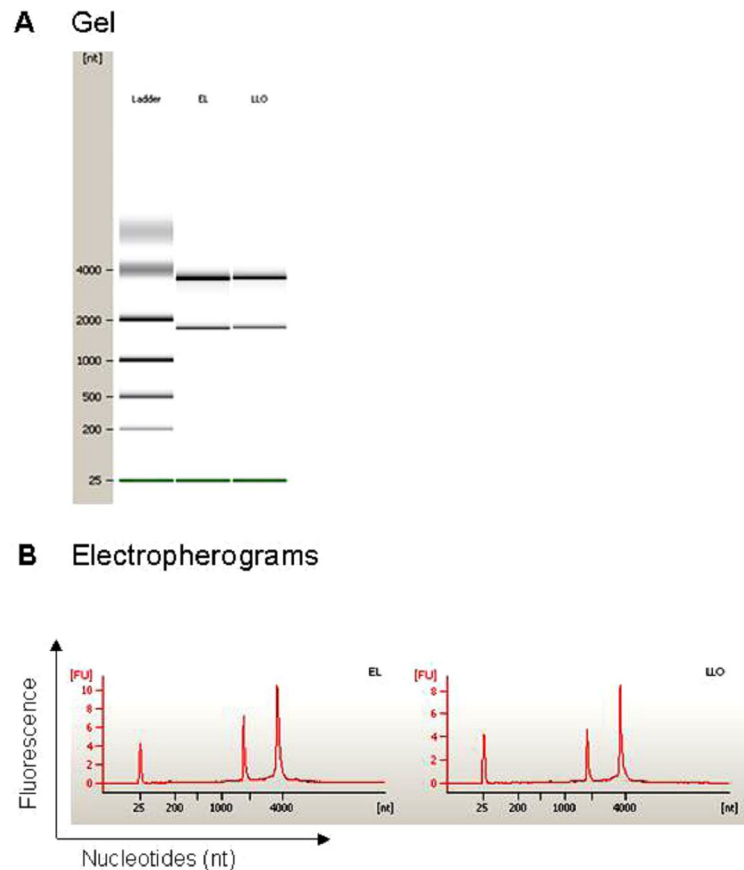
**Figures 22. Simultaneous Treg suppression/proliferation assay.** CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from *E. coli*- or *E. coli*-LLO- vaccinated animals induce different levels of suppression. Mice received three s.c. injections of *E. coli* or *E. coli*-LLO and splenocytes were harvested 7 days after the last injection. All the pooled splenocytes were separated into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. In a MLR, CD4<sup>+</sup>CD25<sup>+/High</sup> (variable number) were cultured with CD4<sup>+</sup>CD25<sup>-</sup> (10<sup>5</sup>) T cells for 4 days in the presence of 0.5 µg/ml anti-CD3 and 2 x 10<sup>5</sup> naive irradiated splenocytes as feeders. Proliferation of responders was measured by adding <sup>3</sup>H-Thymidine for the last 18 h of culture period and inhibition of proliferation was determined. The experiments presented are representative of two independent experiments. \*\**p* < 0.01, \*\*\**p* < 0.001, *E. coli* vs *E. coli*-LLO.

## 4.3 RESULTS

### 4.3.1 RNA Integrity

The results of the Bioanalyser run are visualised in a gel image and an electropherogram (Figure 23) using the Agilent 2100 Bioanalyzer expert software. In addition to this visual control, the software allows the generation of a RNA Integrity Number (RIN) to check integrity and overall quality of total RNA samples. The RIN value is calculated by a proprietary algorithm that takes several QC parameters into

account, for example, 28S RNA/18S RNA peak area ratios and unexpected peaks in the 5S RNA region (Fleige and Pfaffl 2006). Here, both RNA samples (*E. coli* and *E. coli*-LLO) revealed RIN values between 9.3 and 9.6 and RNA yields between 1.7  $\mu\text{g}$  and 2.5  $\mu\text{g}$ .

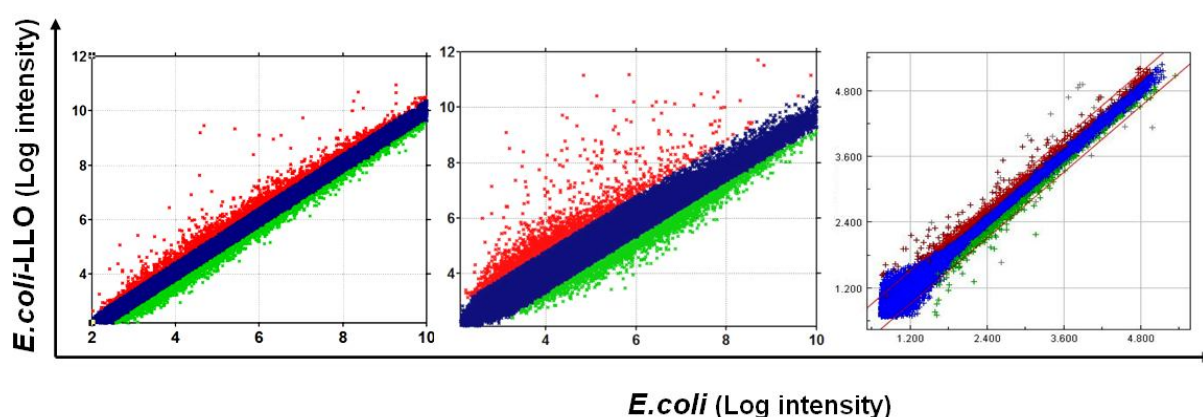


**Figure 23.** Gel image (A) and electropherogram (B) of total RNA samples. As a reference, the RNA molecular weight ladder (in nucleotides, nt) is shown in the first lane. The lowest migrating green band represents an internal standard. The two prominent peaks within the electropherograms represent ribosomal RNA: left 18S RNA, right 28S RNA. Scaling of the y-axis is done automatically, relative to the strongest signal within a single run.

#### 4.3.2 RNA Microarray GeneChip Analysis

1  $\mu\text{g}$  of total RNA sample in each group was used as starting material, reverse transcribed to cDNA and transcribed *in vitro* to cRNA. 15  $\mu\text{g}$  of cRNA in adjusted

quantity was labeled with biotin, fragmented, and hybridised to Affymetrix™ GeneChip Mouse Genome 430 2.0 Array. The GeneChip Mouse Genome 430 2.0 Array comprises more than 40,000 transcripts on a single array probe set. All the raw expression values in one genechip were plotted in a single figure. Red and green spots were indicative of expression differences. 2 repetitive analyses were carried out in the same experiment setting (Figure 24).



**Figure 24. Scatter plot of signal intensities of all spots.** The signal intensity of each spot represented by a dot is shown in double logarithmic scale. X-axis: log signal intensity from *E. coli*; Y-axis: log signal intensity from *E. coli*-LLO. Red/green spots define the areas of 2-fold differential signal intensities. Blue spots: unchanged genes. Each figure represents an separate microarray analysis. The first and second experiments were performed in the Institute of Cancer, Queen Mary University of London, and analysed by Bioconductor and R Statistical software (with the assistance of Dr. Claude Chelala). The data were plotted by GraphPad Prism 5.0. The third experiment was performed at the microarray technical platform, Université de Nantes, Faculté de Médecine, Nantes, France. The data were processed by Feature Extraction Software (FES) and Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware) with the assistance of Dr. Bernhard Gerstmayer (Miltényi Genomic Services).

Data were analysed as described in the **Data processing section (2.17.3)** and compared to previously published data available from the GEO database (Fontenot,

Rasmussen et al. 2005) (RNA from CD4<sup>+</sup>CD25<sup>High</sup>Foxp3<sup>+</sup> cells as the naïve group, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM44980>).

Regarding the functional impact and reversal of Treg suppression, my interests are focused on the genes which are related to inflammation, cytokines/chemokines, growth factors, transcriptional factors, and otherwise genes involved in immune regulation. A comprehensive literature review was also performed to see if these genes are involved in immune augmentation/tolerance. I focused on genes reported as significantly up- or down- regulated in two consecutive microarray analyses with literature-accessible evidence. Table 8 shows the genes I selected which could be responsible for the functional reversal of Treg (25 up-regulated and 13 down-regulated genes).

**Table 8 List of differentially expressed genes in Non-functional Treg**

Upregulated Genes	
Inflammation-associated genes	S100 calcium binding protein A8 (Calgranulin 8)
	S100 calcium binding protein A9 (Calgranulin 9)
	Lipocalin-2 (LCN-2)
	Cathelicidin antimicrobial peptide (Camp)
	Secretory Leukocyte protease inhibitor (SLPI)
Cytokine and chemokines genes	IL-1 $\beta$ (IL1b)
	IL-17
	IL-22
	IL-10
	chemokine (C-C motif) receptor 1 (CCR1)
	chemokine (C-X-C motif) ligand 2 (CXCL2)
	transforming growth factor- beta induced (TGF- $\beta$ i)
Growth factor genes	Amphiregulin (AmR)
Surface marker/ protein	CD24a antigen (CD24a)
/ receptor	Tumour necrosis factor receptor superfamily, member

	13c (Tnfrsf13c, CD268) Membrane-spanning 4-domains subfamily A (Ms4a1) Neutrophilic granular protein (NGP) Interferon induced transmembrane protein 6 (Ifitm6)
Cytoplasmic protein / Metabolism	Granzyme B (GzmB) Lactotransferrin (Ltf) Cathepsin H (Ctsh)
Others	Lysozyme (Lsz) Chitinase 3-like 3 (C3L3) Immunoglobulin heavy chain 6 (Igh6) B lymphoid kinase (BLK)

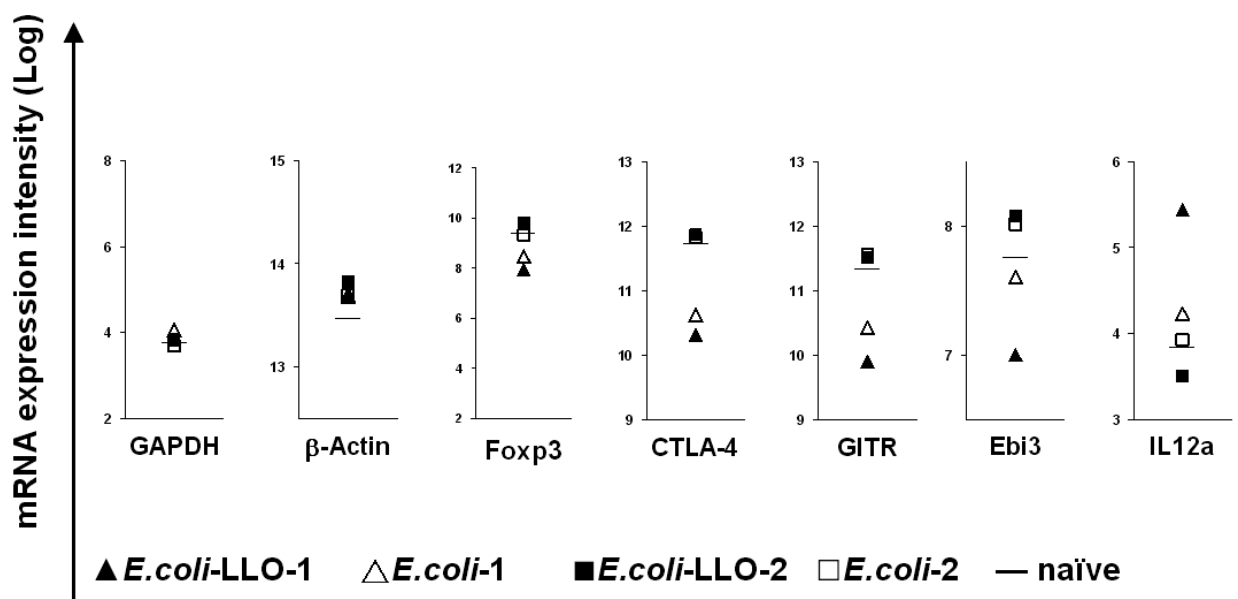
#### Down regulated genes

Tumour necrosis factor (alpha-induced protein 1) (Tnfaip1)  
Tumour necrosis factor superfamily, member 11 (Tnfsf11, CD254)  
Heat shock protein 1a (Hspa1a)  
Prostaglandin E receptor 1 (subtype EP1) (Ptger1)  
Extracellular proteinase inhibitor (Expi)  
Apoptosis, caspase activation inhibitor (Aven)  
DAZ interacting protein 1 (Dzip)  
CD 28 antigen (CD28)  
Proteasome activator subunit 4 (Psme4)  
Basic transcription factor 3 (Btf3)  
src family associated phosphoprotein 1 (Skap1)  
Glyoxalase 1 (Glo1)  
Eosinophil-associated ribonuclease 3 (Ear3)

#### 4.3.3 Expression of Control Genes in Each Group

In the reference of commonly-used housekeeping genes, both samples showed equivalent RNA quantity in the hybridisation reaction. Here I also compared the expressions of other control genes which are the well-recognised Treg phenotype

markers (Figure 25): Forkhead box P3 (Foxp3), Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), and Glucocorticoid-induced TNF receptor (GITR, as known as Tnfrsf18). In addition, IL-35, which is an Ebi3-IL-12 $\alpha$  heterodimeric cytokine, is recently proposed as a novel inhibitory cytokine specifically produced by Treg cells and is required for maximal suppressive activity (Vignali, Collison et al. 2008). We can see the near equivalent RNA amount in each group from the usual housekeeping genes. From the phenotype marker genes for Treg, variable expression with minimal difference was seen but not consistently limited to one specific group. Additionally, some large variations observed between the repetitive experiments (eg, CTLA-4 and GITR) may have been due to experimental differences or the primary nature of the cells. Such variation may limit the interpretation of subsequent results and the data obtained need to be further validated.



### Internal Control Genes

**Figure 25. Housekeeping genes and common Treg phenotype markers expression.** Housekeeping genes and common Treg phenotype markers were expressed between the CD4<sup>+</sup>CD25<sup>High</sup> cells from *E. coli*- and *E. coli*-LLO-immunised mice. 2 separate microarray data were shown in RNA expression intensity (Log).

#### 4.3.4 Expression of up-regulated genes

Significantly upregulated genes in *E. coli*-LLO Treg were demonstrated in Figure 26. The gene expression difference ranged from 0.3 to 6 log. When compared to the gene expression in naïve Treg, most of genes were at a similar level to Treg from *E. coli* group (except IL-22, IL-17A, CXCL2, SLPI, Igh6, and BLK). Notably, S100 A8/A9, IL-17A, C3L3, LSZ, LCN2, NGP, and Ltf were up-regulated throughout 3 repetitive microarray analyses.

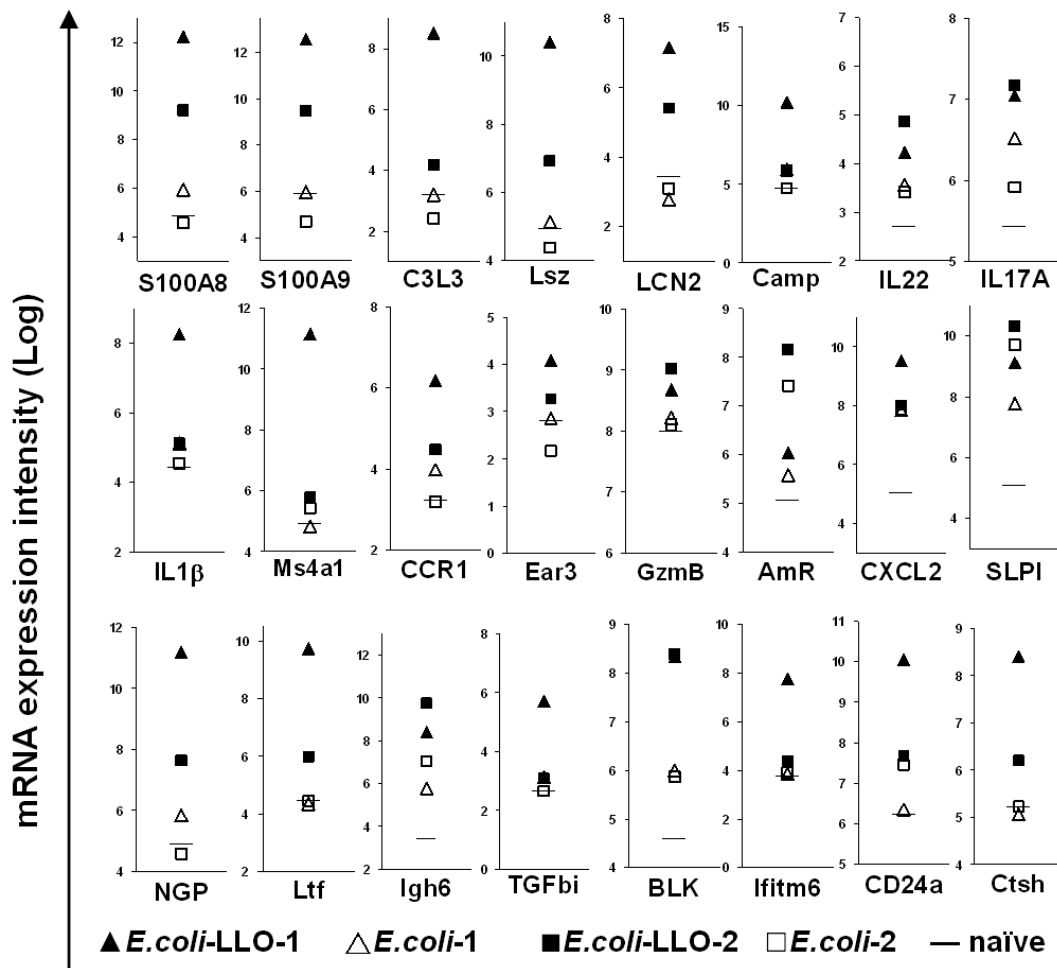


Figure 26. Up-regulated genes expressed in the Treg from *E. coli*-LLO-immunised mice compared to *E. coli*. Data were plots by 2 microarray analyses.



### 4.3.5 Expression of down-regulated genes

Significantly down-regulated genes in *E. coli*-LLO Treg (compared to *E. coli*) are shown in Figure 27. The gene expression difference ranged from 0.3 to 1.5 log. Only Hspa1a was found to be consistently down-regulated throughout 3 microarray analyses.

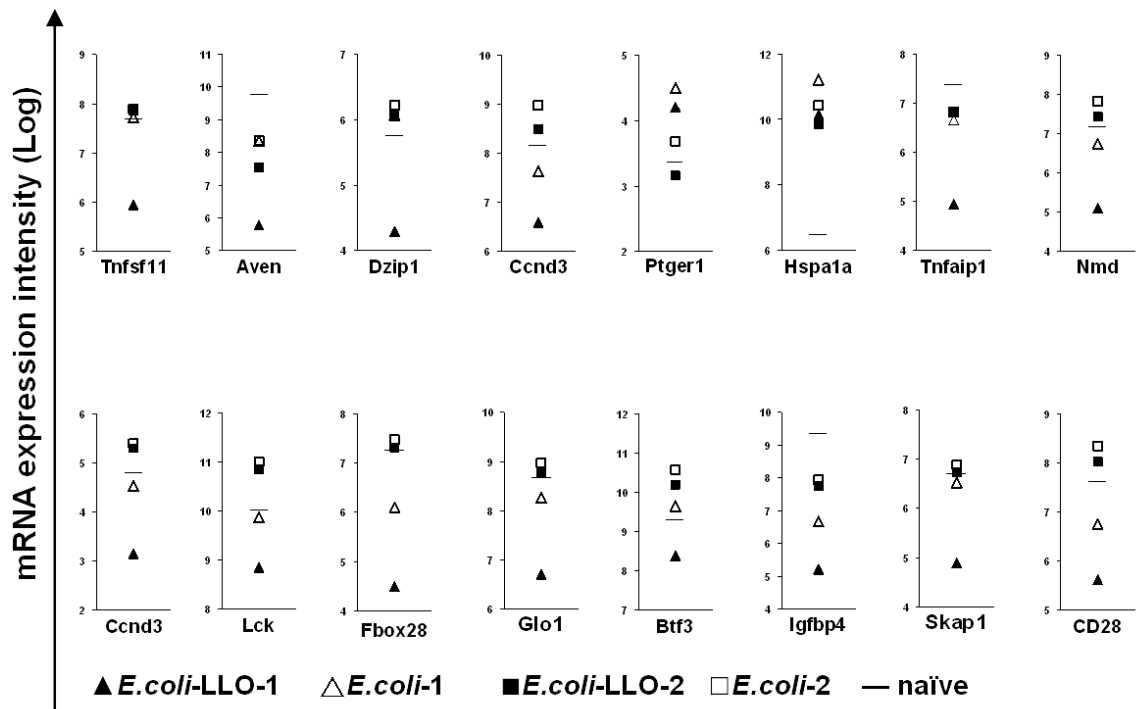
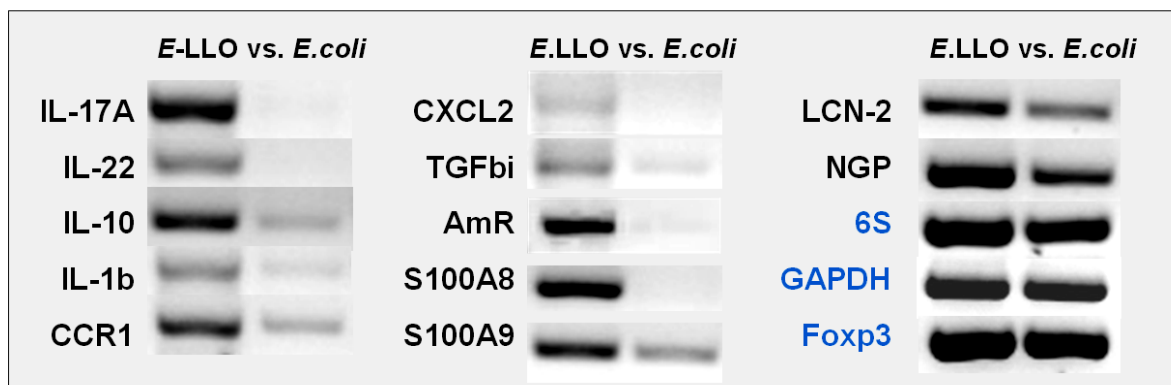


Figure 27. Down-regulated genes expressed in the Treg from *E. coli*-LLO-immunised mice compared to *E. coli*. Data were plots by 2 microarray analyses.

### 4.3.6 Verification of Microarray Data by RT-PCR

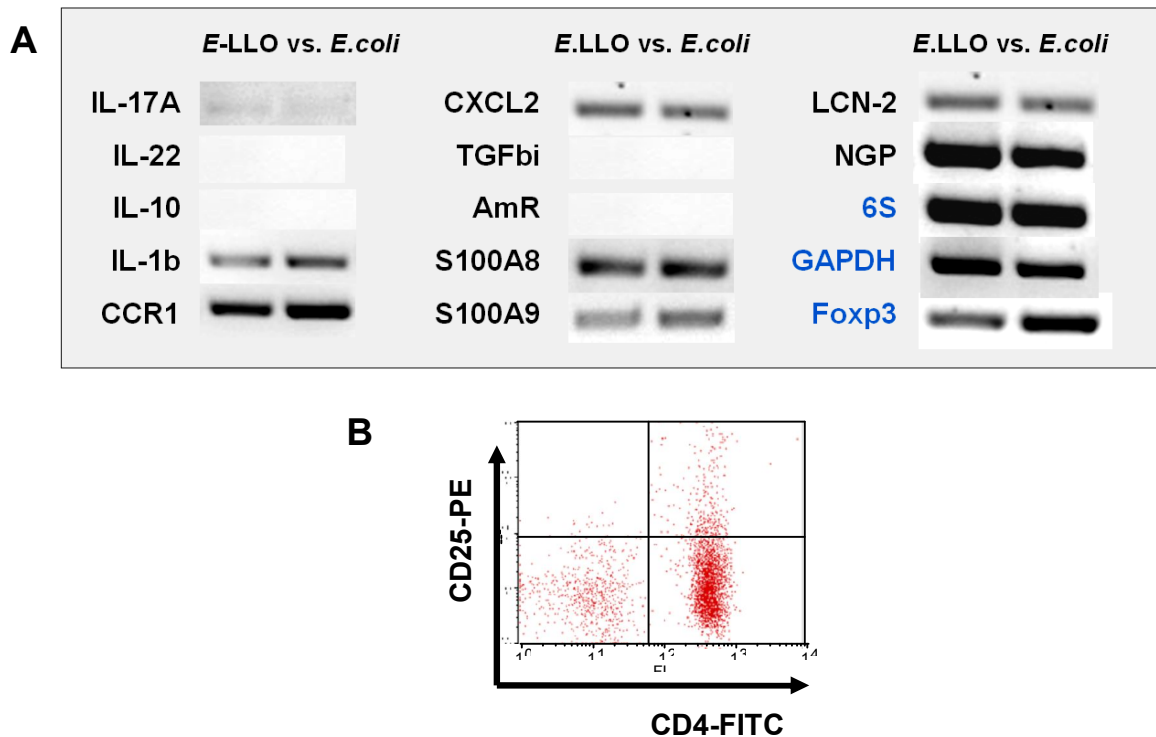
The genes selected from microarray data were verified by PCR following reverse transcription of total RNA extracted from different batches of immunised mice (*E. coli* and *E. coli*-LLO). While 6S is routinely used as an endogenous control for cDNA quantity, it may not be an ideal internal standard under conditions of variable lymphocyte activation. Therefore in addition to 6S, GAPDH was also used as an

internal reference. More importantly, I also included Foxp3 as an additional internal control since this is a transcriptional factor specific for the suppressive Treg and the Foxp3<sup>+</sup> Treg prevalence did not change by the bacterial vaccine in previous experiments. Parts of the up-regulated genes were verified by RT-PCR (Figure 28) and most of them were cytokines/chemokines (IL-17A, IL-22, IL-10, IL-1 $\beta$ , CCR1, CXCL2, TGF- $\beta$ i), growth factor (AmR) or inflammatory-related genes (S100A8/A9, LCN-2, NGP). Meanwhile, CD4<sup>+</sup>CD25<sup>-/Low</sup> cells, which were separated during the Treg sorting, were used for a comparison in the verification (Figure 29). In those CD4<sup>+</sup>CD25<sup>-/Low</sup> cells, most of the genes were either less significantly expressed or not differentially expressed between *E. coli* and *E. coli*-LLO group. Notably, Foxp3 expression was detected by PCR in both groups, which could be attributed to the presence of CD4<sup>-</sup> Treg, trace CD25<sup>+</sup> Treg, or some activated CD4<sup>+</sup>CD25<sup>-</sup> cells expressing Foxp3.



**Figure 28. Verification of the differentially expressed genes by RT-PCR.**

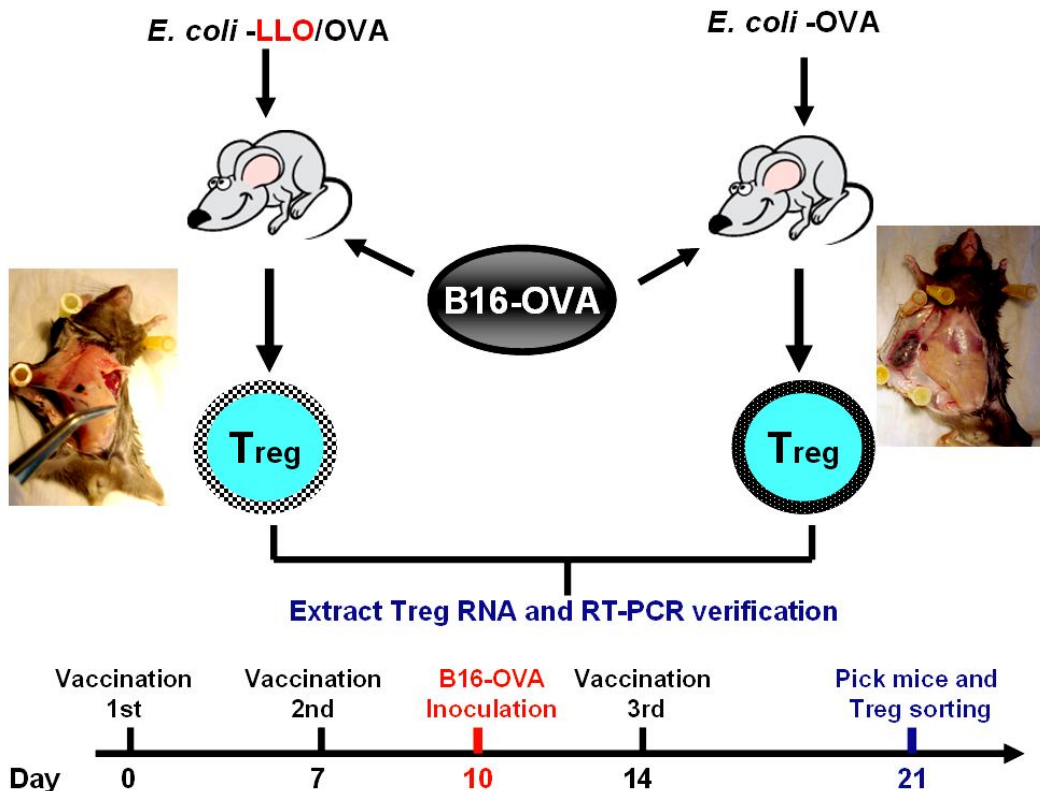
10 Mice were immunised 3 times with *E. coli* or *E. coli*-LLO at one-week intervals and CD4<sup>+</sup>CD25<sup>High</sup> Treg were purified from pooled splenocytes one week after the last vaccination.



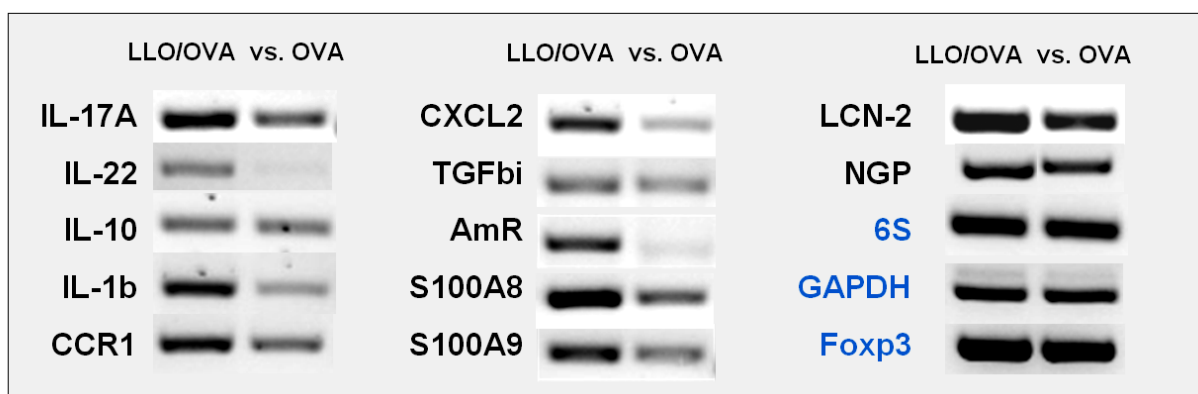
**Figure 29.** (A) Comparison of the selected genes expression in CD4<sup>+</sup>CD25<sup>-/Low</sup> (Tconv) cells which were prepared at the same time as Treg were sorted. (B) The purity of isolated Tconv cells was analysed by FACS (>85%)

#### 4.3.7 Repeat RT-PCR Verification in the Tumour-harboured Mice

In an attempt to better visualise the differentially expressed genes which could be another functional determinant of Treg, I compared the RNA expression between Tregs from mice that received *E. coli*-LLO/OVA vaccination and rejected the tumours and Tregs from mice that received *E. coli*-OVA and were unprotected (Figure 31). Most of the expression differences remained valid (as *E. coli* vs. *E. coli*-LLO) except IL-10 and TGF-βi.



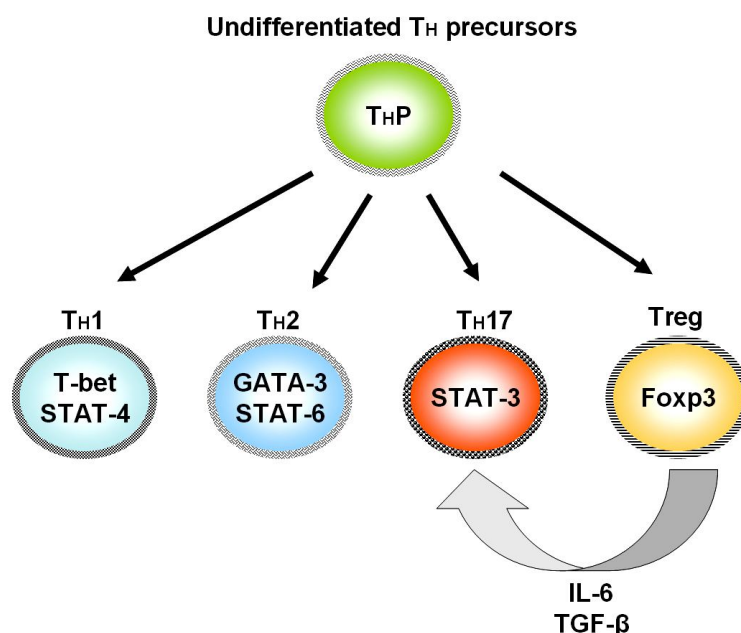
**Figure 30. Schematic representation of RT-PCR from Treg in tumour-harboured mice.** 15 mice in each group were immunised with *E. coli*-OVA or *E. coli*-LLO/OVA on day 0, 7 and 14.  $10^6$ /mouse B16-OVA cells were inoculated between the 2<sup>nd</sup> and 3<sup>rd</sup> vaccinations (day 10) and mice were chosen from each group based on the performance of tumour protection. 10 mice were chosen in each group with near- or completely tumour-rejection in the *E. coli*-LLO/OVA group and significantly advanced tumour growth in the *E. coli*-OVA group. Total RNA was extracted from purified Treg ( $CD4^+CD25^{high}$ ).



**Figure 31. RT-PCR comparison of the expression of selected genes in  $CD4^+CD25^+$  cells** which were purified from tumour-established mice as previously described in Figure 29.

#### 4.3.8 Analysis of Committed helper T cell-related Transcriptional Factors

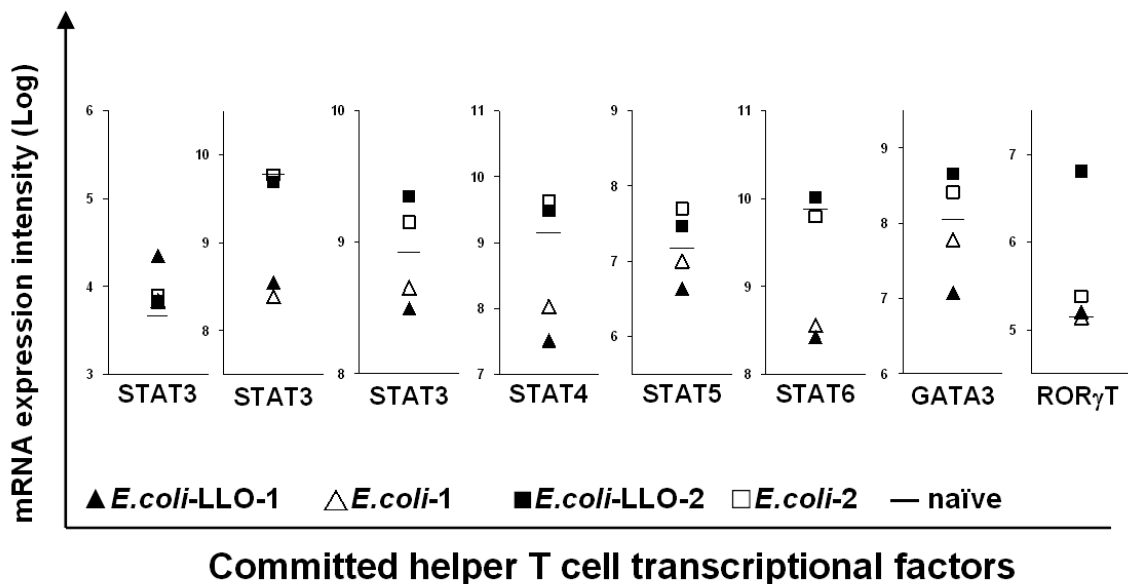
Uncommitted (naive) murine CD4<sup>+</sup> T helper cells (Thp) can be induced to differentiate towards T helper 1 (Th1), Th2, Th17 and Treg phenotypes according to the local cytokine milieu (Afzali, Lombardi et al. 2007; Zhu and Paul 2008). This can be demonstrated most readily both *in vitro* and *in vivo* in murine CD4<sup>+</sup> T cells. The presence of IL-12 (signalling through signal transduction and activator of transcription STAT-4) skews towards Th1, IL-4 (signalling through STAT-6) towards Th2, TGF- $\beta$  towards Treg and IL-6 and TGF- $\beta$  towards Th17. The committed cells are characterized by expression of specific transcription factors, T-bet for Th1, GATA-3 for Th2, Foxp3 for Tregs and ROR $\gamma$ T for Th17 cells (Figure 32). Since the Treg obtained from *E. coli*-LLO/OVA-immunised cells could demonstrate IL-17A gene expression, it is crucial to examine the expression of relevant transcription factors specific for Th17..



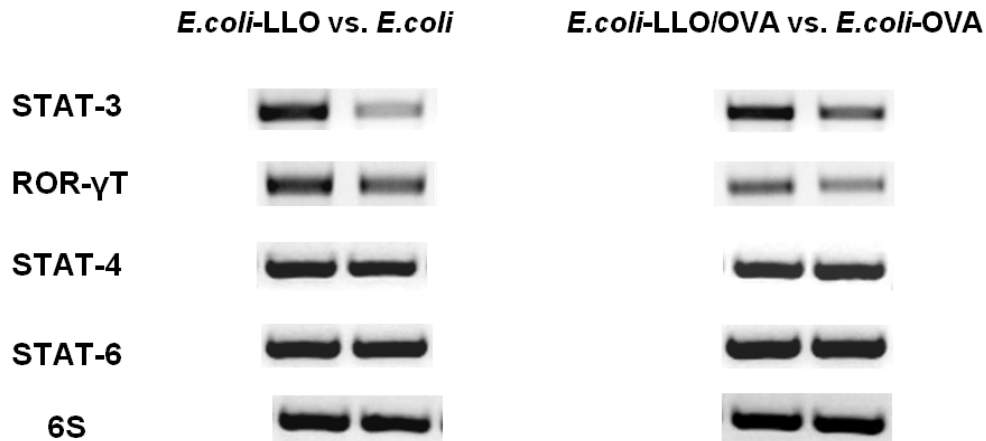
**Figure 32. T helper cell commitment towards specific lineages in mice.** T helper cell precursors (ThP) can be skewed toward mutually exclusive Th1, Th2, Th17 and Treg phenotypes on the basis of the cytokine environment. Development of Th17 and Treg

phenotypes both require the presence of TGF- $\beta$  but the presence of IL-6 preferentially skews the response towards a Th17 phenotype, Treg are characterised in mice by expression of Foxp3.

In Figure 33, the RNA expression intensities of STAT-3 (3 variants). STAT-4, STAT-5, STAT-6, GATA-3, and ROR $\gamma$ T were plotted. None of these transcription factors demonstrated significantly different expression in microarray analysis. However, in RT-PCR (Figure 34), STAT-3 showed slightly higher expression in *E. coli*-LLO- or *E. coli*-LLO/OVA-vaccinated Treg.



**Figure 33.** RNA expression of helper T cell transcription factor genes. Data were plots by 2 microarray analyses.



**Figure 34.** RT-PCR of the genes relevant to transcription factors of committed helper T cells.

#### 4.4 CONCLUSION

I have used the genome-wide screening of the conditioned-Treg RNA as the first step with an attempt to unravel the underlying mechanisms of how *E. coli*-LLO vaccine affected regulatory immune responses. Many upregulated and downregulated genes were found within the categories of cytokine/chemokines, inflammation-related, growth factors, or surface/cytoplasmic proteins. Apart from specific gene expression, some changes of the transcription factors of committed T helper cells were also demonstrated but the nature of these changes needs to be examined further.

# **CHAPTER 5: IS *E. COLI*-LLO ACTIVE AGAINST A “REAL” TUMOUR ANTIGEN? WILMS’ TUMOUR ANTIGEN-1 AS AN EXAMPLE**

## **5.1 INTRODUCTION**

Antigens, whether “shared” or “unique” tumour antigens, used in cancer vaccines should preferably be molecules that are different between normal cells and tumour cells, ensuring that the immune response generated by vaccination will target for destruction antigen-bearing tumour cells but spare normal cells. In cancer, most antigens are derived from mutated or modified self-proteins against which there is often a certain level of immune tolerance. This creates particular challenges for the appropriate design of vaccines that have to overcome this tolerance in order to elicit anti-tumour immunity with limited autoimmunity. The *E. coli*-LLO vaccine expressing model antigen, OVA, has demonstrated its efficacy. However, OVA is a non-self antigen in mice, it is therefore required to determine the effect and toxicity of this vaccine expressing a real tumour antigen. A very large number of tumour antigens have been characterised to date and most of them are melanocyte differentiation antigens or tumour-specific mutated gene products, which are not expressed in normal tissues. Therefore, we are keen to choose an oncofetal protein, expressed differentially in tumour and normal tissues. In the collaboration with Prof. HJ Stauss (Royal Free Hospital), we chose Wilms’ tumour antigen-1 (WT1) as our experimental tumour antigen. This choice was mainly dictated by the availability of a peptide library covering the whole coding sequence, allowing the determination of the antigenic motif involved.



### **5.1.1 WT1 proteins: functions in growth and differentiation**

Wilms' tumour or nephroblastoma is a paediatric kidney malignancy that was first described by Max Wilms in 1899. So far, the Wilms' tumour 1 gene (WT1) at 11p13 is the only gene involved in development of Wilms' tumour that has been cloned and initially classified as a tumour suppressor gene. It is now recognised that WT1 is homozygously mutated in 5–10% of Wilms' tumours. The WT1 protein has 4 different isoforms due to alternatively splicing of exon 5 and 9 in mammals (Call, Glaser et al. 1990).

Extensive structure and function analyses of the mammalian WT1 protein have been performed by many groups. From the primary structure of the WT1 proteins, it was predicted that they could function as transcription factors. They may also be involved in post-transcriptional regulation of target genes because of a potential RNA recognition motif (Kennedy, Ramsdale et al. 1996). A number of putative target genes, comprising growth factor genes/receptors, transcription factors genes, and protein-encoding genes have been identified previously (Scharnhorst, van der Eb et al. 2001). In addition, WT1 is also known to bind to several other proteins, which are also transcription factors and/or alter the transcriptional regulatory properties of WT1.

WT1 plays an essential role in urogenital development and is expressed during all stages of kidney development, especially in differentiation of the metanephric mesenchyme (Pritchard-Jones, Fleming et al. 1990). It has been demonstrated that WT1 can induce features of renal epithelial differentiation in mesenchymal fibroblasts (Hosono, Luo et al. 1999). Wilms' tumour is thought to arise from the condensed metanephric mesenchyme that is destined to differentiate into the epithelial components of the nephron but fails to do so properly and instead continues to

proliferate (Hastie 1994). Apart from the urinary system during embryogenesis, WT1 is expressed in the adult, but only in low amounts in the nuclei of some normal tissues, such as early haematopoietic precursor, kidney, and gonadal cells (Baird and Simmons 1997).

### **5.1.2 The oncogenic role of WT1**

Recent studies demonstrated that the WT1 gene is overexpressed in most types of adult leukaemia, including acute myeloid leukaemia (AML), chronic myeloid leukaemia, and acute lymphocytic leukaemia (ALL) and in some patients with myelodysplastic syndromes (MDS) (Miwa, Beran et al. 1992; Miyagi, Ahuja et al. 1993; Inoue, Sugiyama et al. 1994; Keilholz, Menssen et al. 2005). WT1 is also expressed in many types of lung, thyroid, breast, testicular, and ovarian carcinomas and in melanoma (Bruening, Gros et al. 1993; Silberstein, Van Horn et al. 1997; Oji, Ogawa et al. 1999). WT1 overexpression represents the result of several factors, including the occurrence of genetic damage on the progenitor cell compartment. An *in-vitro* study (Inoue, Tamaki et al. 1998) showed that increased WT1 expression can block normal differentiation and enhance proliferation of haematopoietic progenitor cells, implicating the potential of WT1 contributing to leukaemogenesis. For MDS patients, a significant correlation was found between WT1 expression levels, blast cell percentage, and the presence of cytogenetic abnormalities. There is also a significant correlation between the amount of WT1 transcripts and the IPSS score, which currently represents the most reliable risk index of disease progression (Spanaki, Linardakis et al. 2007). In patients with leukaemia, solid tumours or soft tissue sarcoma, the level of WT1 expression correlates with the clinical tumour

progression and also the prognosis (Oji, Miyoshi et al. 2002; Chiusa, Francia di Celle et al. 2006; Netinatsunthorn, Hanprasertpong et al. 2006; Sotobori, Ueda et al. 2006). Furthermore, RT-PCR-based quantification of WT1 mRNA may serve as marker for minimal residual disease monitoring in AML or following bone marrow transplantation (Ogawa, Tamaki et al. 2003; Weisser, Kern et al. 2005). Osaka et al demonstrated that WT1 expression was detected in most 7,12-dimethylbenzanthracene-induced erythroblastic leukaemias and a tendency for cells with high levels of WT1 expression to turn into leukaemic cells (Osaka, Koami et al. 1997). The growth of leukaemic and solid tumour cells could be inhibited by treatment with WT1 antisense oligomers (Yamagami, Sugiyama et al. 1996; Glienke, Maute et al. 2007).

In summary, WT1 has received increasing attention as a candidate for immunotherapy based on previous experiments and preclinical studies. WT1 vaccination by means of bacterial vehicle expressing LLO to enhance antigen presentation has not been studied in the past. In this context, we may observe the immune response from this natural adjuvant and the advantage of LLO in modifying antigen presentation pathway.

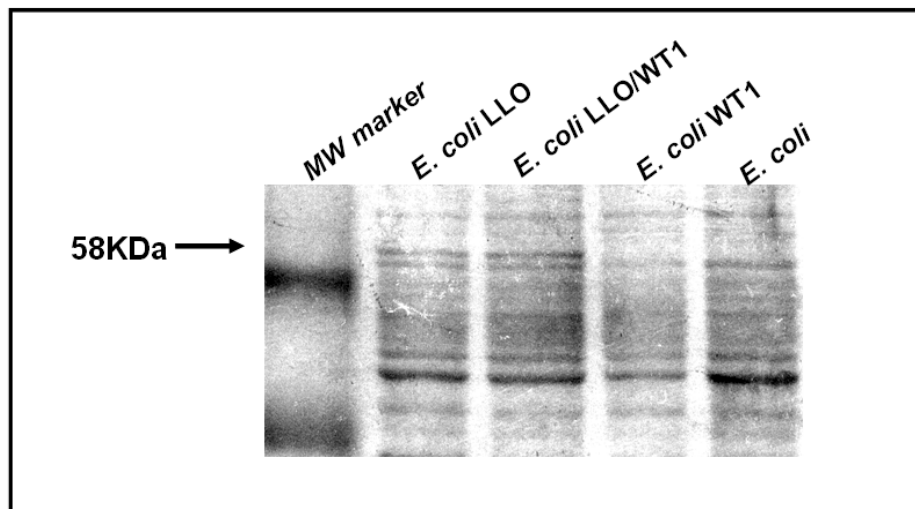
## **5.2 RESULTS**

### **5.2.1 Expression of Target protein in *E. coli* (MC4100/DE3)**

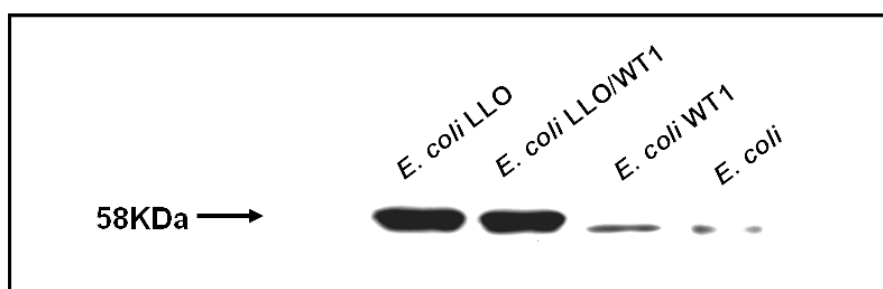
#### **5.2.1.1 Expression of LLO**

The expression of LLO in *E. coli* harbouring the plasmid pDP-E3615, which encodes listeriolysin-O lacking its secretion signal under the control of the constitutive *tet* promoter, was first analysed. Coomassie brilliant blue staining of the

gel (Figure 35) visualised a band at 58 kDa in *E. coli*-LLO and *E. coli*-LLO/WT1, which was absent in either *E. coli*-WT1 or *E. coli*. This observation was confirmed by Western Blot using an anti-LLO antibody (Figure 36). These results demonstrated the expression of LLO in pDP-E3615 transformed *E. coli*.



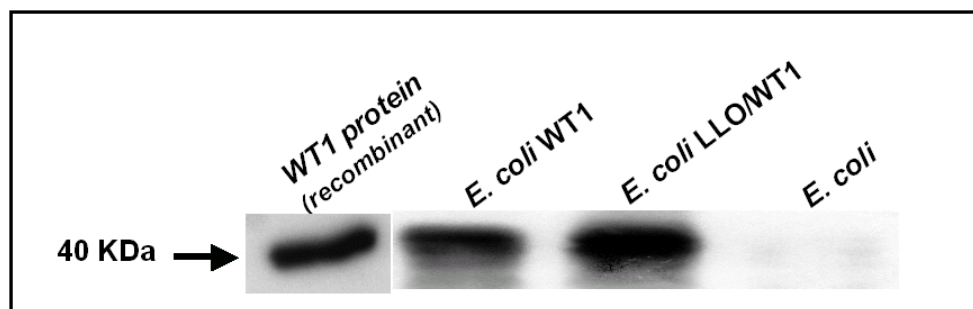
**Figure 35. LLO protein expression in IPTG-induced bacteria.** Proteins from  $10^8$  bacteria were resolved on 10% SDS–polyacrylamide gels and visualised by Coomassie brilliant blue staining. Migration of molecular weight standards is shown to the left of each panel in kDa.



**Figure 36. Detection of LLO expression in Western Blot by specific antibody.** (primary) LLO-specific antibodies (1/2000, Diatheva, Italy) and (secondary) polyclonal anti-mouse immunoglobulins/horseradish peroxidase (HRP)-conjugated antibody (1/5000, DakoCytomation, U.S.) were used to detect the presence of LLO in IPTG-induced bacteria.

### 5.2.1.2 Expression of WT1

The expression of WT1 in *E. coli* harbouring the plasmid pCRT7/CT, which encodes 40 kDa truncated WT1 protein under the control of Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG)-inducible T7 phage promoter, was analysed by Western Blot. Figure 37 demonstrates the presence of WT1 in the relevant bacteria.

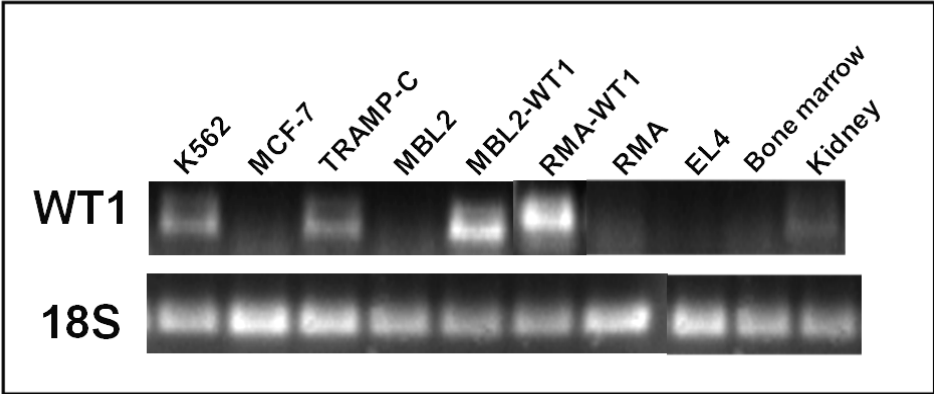


**Figure 37. WT1 expression in IPTG-induced bacteria.** Detection of WT1 expression in protein obtained from  $10^8$  bacteria by (primary) monoclonal mouse anti-human Wilms' tumour 1 protein (6F-H2 clone) antibodies (1/1000, DAKO, California, USA) and (secondary) polyclonal anti-mouse immunoglobulins/horseradish peroxidase (HRP)-conjugated antibody (1/2000, DakoCytomation, USA) and revealed by ECL (GE Healthcare, UK).

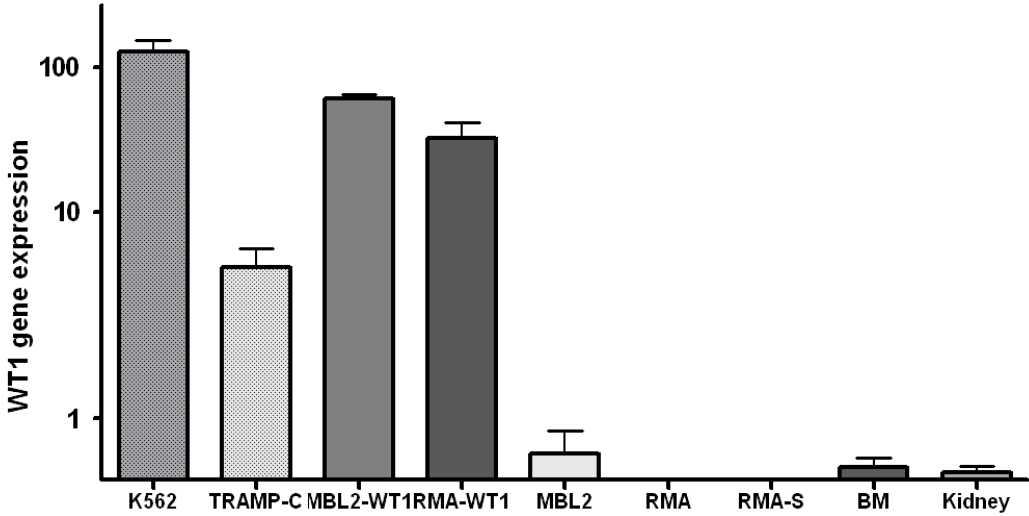
### 5.2.2 WT1 mRNA expression

RT-PCR and quantitative real-time PCR were carried out to determine the WT1 mRNA expression in various tumour cell lines and normal tissues. K562 cells (derived from a CML patient in blast crisis), TRAMP-C (TRansgenic Adenocarcinoma of the Mouse Prostate, a transgenic line of C57BL/6 mice), WT1 plasmid transfected MBL2 (MBL2-WT1, originated from a Moloney virus-induced C57BL/6 lymphoma) and RMA (RMA-WT1, originated from a Rauscher virus-induced C57BL/6N T cell lymphoma) were shown to express WT1. A trace of WT1 expression was seen in

normal mouse kidney tissue (Figures 38 and 39). From these experiments, TRAMP-C and MBL2-WT1 were chosen to test our *in-vivo* tumour challenge model following vaccination.



**Figure 38. WT1 RNA expression in tumour cell lines and normal tissues by RT-PCR.** The amplified WT1 product is 147 bp long. The RNA of the housekeeping 18S gene was amplified to indicate the amount of RNA in each sample. The 18S product is -200 bp long.

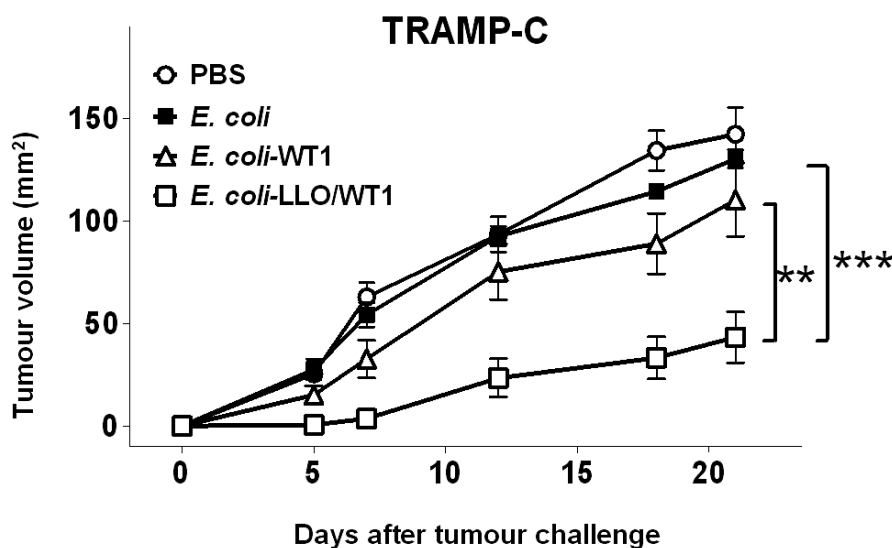


**Figure 39. WT1 RNA expression in tumour cell lines and normal tissues by qRT-PCR.** Total mRNA from BM, kidney cells, and cell lines were reverse transcribed to cDNA, and WT1 gene expression was determined by real-time RT-PCR. Results are expressed relative to 100-fold dilution of the total RNA from K562 cells. Mean values of triplicates are depicted. The RNA of the reference 18S gene was amplified to normalise the amount of RNA in each sample.

## 5.2.3 Tumour protective effect in immunised mice

### 5.2.3.1 Challenge with WT1 naturally-expressed tumour cells

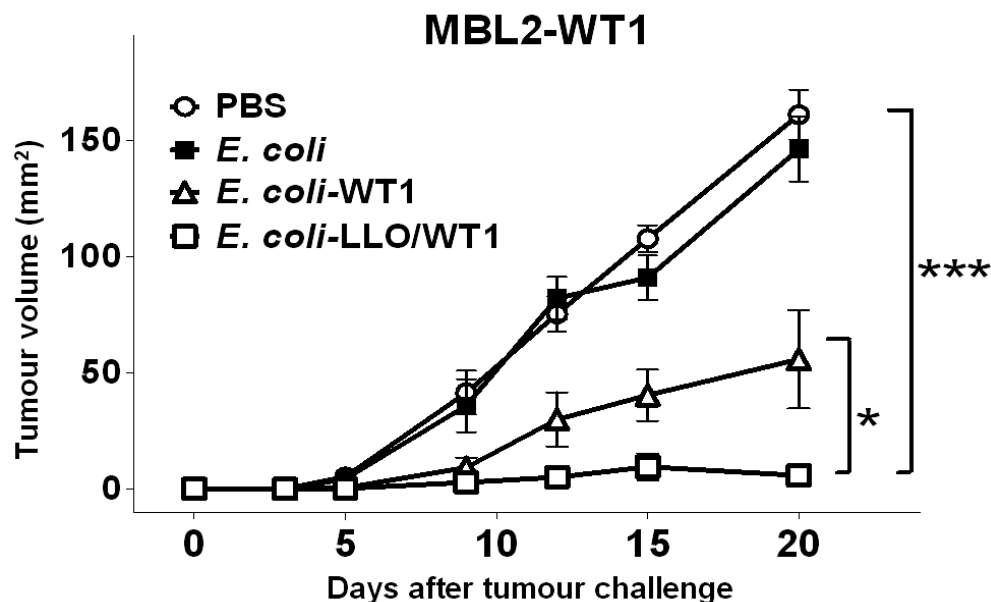
Male C57BL/6 mice received 3 subcutaneous injections of paraformaldehyde-fixed bacteria ( $10^8$ /mouse) at one-week interval then were challenged with TRAMP-C ( $10^6$  cells/mouse) one week after the last vaccination (7 mice in each group). In mice vaccinated with *E. coli*-LLO/WT1, the tumour growth was significantly reduced compared to *E. coli* or *E. coli*-WT1 (Figure 40). There is no significant difference between the groups of *E. coli* and *E. coli*-WT1. In the mice immunised with *E. coli*-LLO/WT1, even though the tumour outgrowth was significantly inhibited at earlier time-point, most of these mice finally had to be sacrificed after 30 days due to advanced tumour.



**Figures 40. Protection of TRAMP-C tumour cells challenge following bacterial vaccinations.** Male C57BL/6 mice received 3 subcutaneous injections of fixed bacteria ( $10^8$ /mouse) or PBS at one-week intervals then were challenged with TRAMP-C ( $10^6$  cells/mouse) one week after the last vaccination (7 mice in each group). Tumour volume was assessed by the maximal 2-dimension measurement and expressed in mm<sup>2</sup>. Results are expressed as mean  $\pm$  SD. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$

### 5.2.3.2 Challenge with WT1-transfected tumour cells

Male C57BL/6 mice received 3 subcutaneous injections of paraformaldehyde-fixed bacteria ( $10^8$  /mouse) at one-week interval then were challenged with MBL2-WT1 ( $5 \times 10^6$  cells/mouse) one week after the last vaccination (7 mice in each group). In mice vaccinated with *E. coli*-LLO/WT1, the tumour growth was significantly reduced compared to the other groups (Figure 41) and most of these mice were long-term protected (>40 days).



**Figures 41. Protection of MBL2-WT1 tumour cells challenge following bacterial vaccinations.** Male C57BL/6 mice received 3 subcutaneous injections of fixed bacteria ( $10^8$  /mouse) at one-week interval then were challenged with MBL2-WT1 ( $5 \times 10^6$  cells/mouse) one week after the last vaccination (6 mice in each group). Tumour volume was assessed by the maximal 2-dimension measurement and expressed in mm<sup>2</sup>. Results are expressed as mean  $\pm$  SD. \*  $p < 0.05$ , \*\*\*  $p < 0.001$

From the results in 5.2.3.1 and 5.2.3.2, the *E. coli* LLO/WT1-vaccinated mice were better protected than *E. coli*-WT1- or *E. coli*-vaccinated ones, implicating that LLO is required for optimal anti-tumour effect. However, the efficacy of long-term tumour

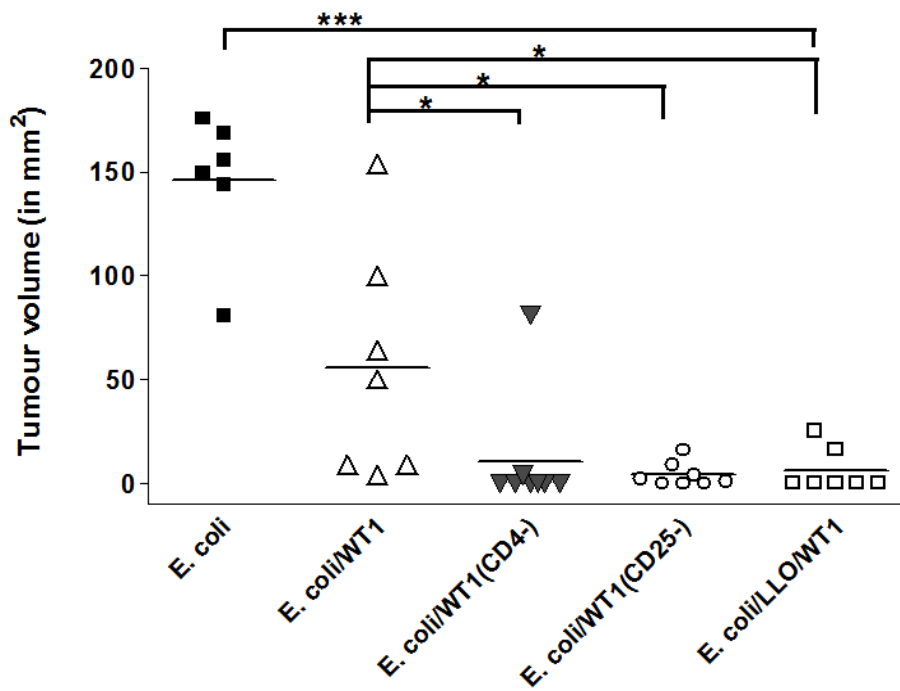
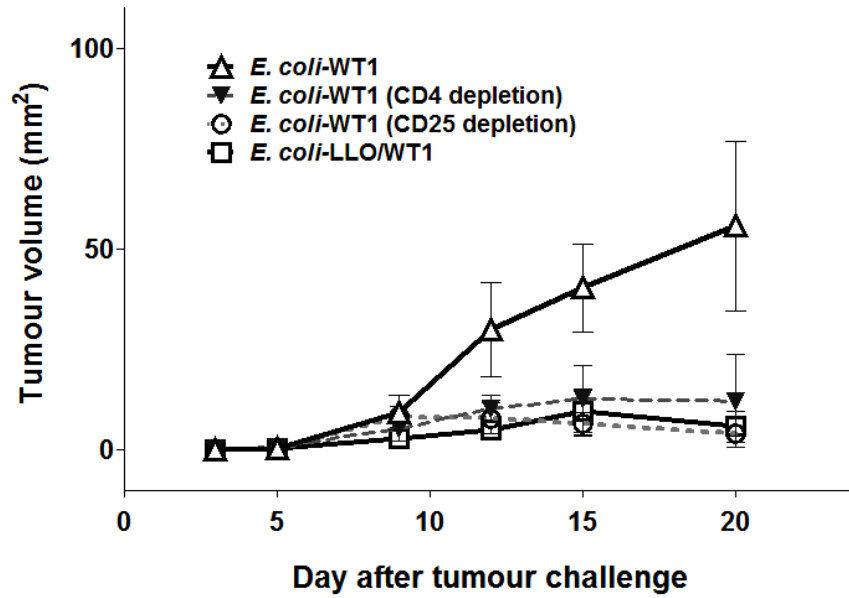


protection by *E. coli*-LLO/WT1 vaccine was different against these two tumour cell lines. This could be attributed to the difference in WT1 expression in tumour cells based on RT-PCR (Figure 39).

#### **5.2.4 The effects of CD4 or CD25 cells depletion**

In previous experiments in Chapter 3.3, I have shown that the removal of CD4<sup>+</sup> cells or CD25<sup>+</sup> cells at the immune priming stage with antibody depletion could enhance the anti-tumour effect of *E. coli*-OVA vaccine. Those data support the conclusion that Treg cells (CD4<sup>+</sup>CD25<sup>+</sup> subset) play an important role in preventing the expansion of OVA-specific CD8<sup>+</sup> T cells following *E. coli*-OVA vaccination and that *E. coli*-LLO vaccine overcame this effect.

To address whether this observation can be extended in the context of WT1 as an antigen, *E. coli*-WT1 vaccinated animals were depleted in CD4<sup>+</sup> or CD25<sup>+</sup> cells during the priming stage and challenged with MBL2-WT1 cells. Figure 42 shows that depletion with either monoclonal antibody resulted in a dramatic increase in *E. coli*-WT1 anti-tumour activity indistinguishable to *E. coli*-LLO/WT1 vaccine. These results suggest that incorporation of LLO in the bacterial vaccine reduces Treg-mediated suppression of CD8<sup>+</sup> T cell expansion and tumour killing effects, as in the case of *E. coli*-OVA.



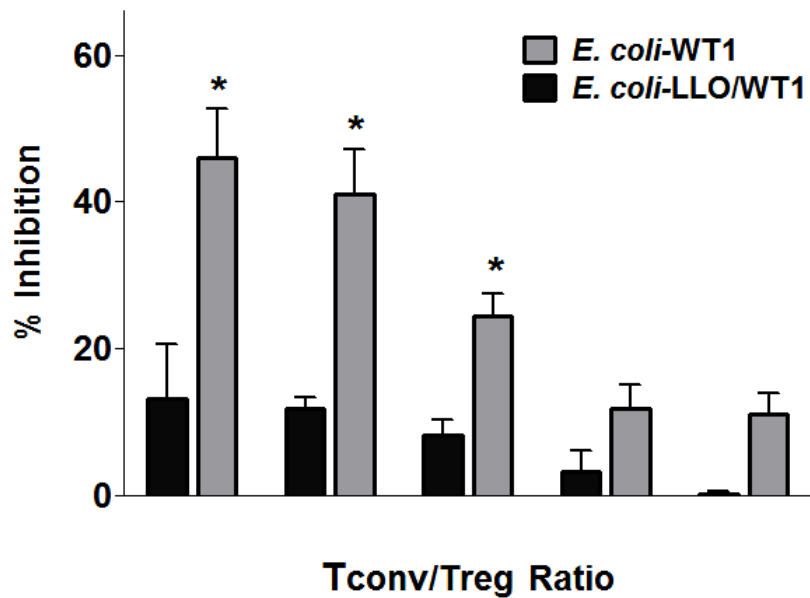
**Figures 42. Tumour protection following CD4+/CD25+ depletion.** (A) In the model of *E. coli* expressing WT1 vaccination with MBL2-WT1 cells challenge, the mice received 3 bacterial vaccinations on days 0, 7 and 14. Depletions were carried out by intra-peritoneal injection of GK1.5 depleting mAb (anti-CD4) (on days -5 and 10) or PC61 mAb (anti-CD25) on day -1 at the stage of T cell priming in the mice with *E. coli* WT1 vaccination. Mice were challenged on day 21 by s.c. injection of  $5 \times 10^6$  MBL2-WT1 cells. The anti-tumour effect in CD4 or CD25 depletion group was similar to the mice vaccinated with *E. coli* LLO/WT1. (B)

The specific time-point tumour measurement was taken on the 20<sup>th</sup> day after MBL2-WT1 challenge. \*\*\*  $p < 0.001$ , \*  $p < 0.05$

### 5.2.5 Assessment of altered Treg function

In Chapter 3.6, animals receiving *E. coli*-LLO or *E. coli*-LLO/OVA vaccines had reduced Treg suppressive function compared to Treg from mice receiving *E. coli* or *E. coli*-OVA vaccines, suggesting that LLO expression in the *E. coli* vaccines dampened the suppressive function of Treg cells.

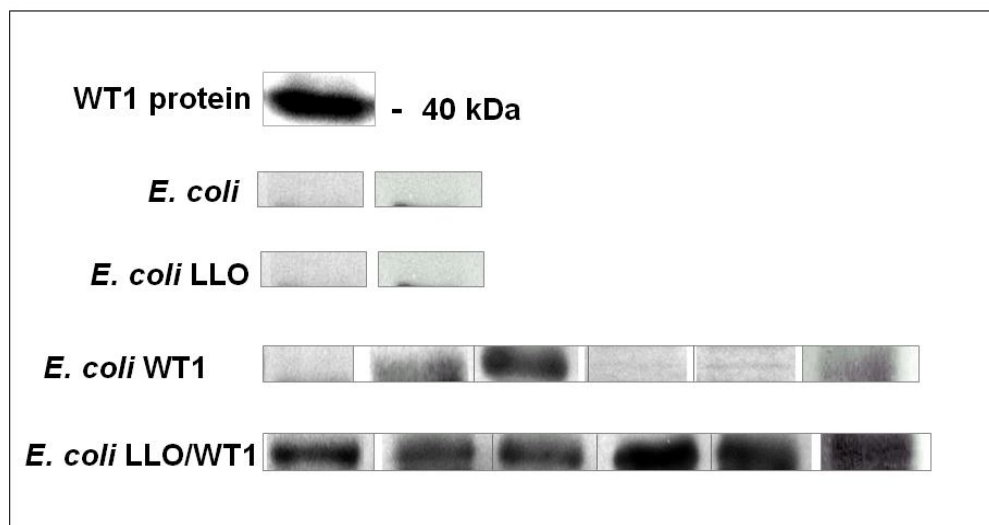
In the *E. coli*-LLO expressing WT1 system, I intended to determine whether the LLO-containing vaccination regimen can elicit a similar effect on the Treg cells function as we observed in *E. coli*-LLO/OVA model. In this experiment, I purified the CD4<sup>+</sup>CD25<sup>high</sup> (Treg) and CD4<sup>+</sup>CD25<sup>low</sup> (Tconv) cells and co-cultured in the presence of anti-CD3 antibody. A 50% inhibition of Tconv proliferation was seen in *E. coli*-WT1 group and 20% in *E. coli*-LLO/WT1 group at 1:1 Treg/Tconv ratio (Figure 43). From this experiment, a similar inhibition effect on Treg cells resulting from incorporation of LLO into vaccine was observed.



**Figure 43. Treg suppression assay in WT1-expressing *E. coli* vaccine model.** Mice received 3 s.c. vaccinations of either *E. coli* WT1 or *E. coli* LLO/WT1 and their splenocytes were harvested 7 days after the last boost injection before being separated into CD4<sup>+</sup>CD25<sup>high</sup> and CD4<sup>+</sup>CD25<sup>low</sup> cells. In a mixed leukocyte reaction, CD4<sup>+</sup>CD25<sup>high</sup> (Treg cells, in variable number) were co-cultured with 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells (Tconv) for 4 days in the presence of 0.5 µg/ml purified anti-CD3 antibody and 2x10<sup>5</sup> naïve irradiated splenocytes. Proliferation of Tconv was measured by the incorporation of <sup>3</sup>H-Thymidine for the last 18hr of culture period. The data were plotted as percent inhibition (of Tconv proliferation) reflecting suppressive functionality of Treg and calculated as [Proliferation (Tconv only)-Proliferation (Treg+Tconv)]x 100%/[Proliferation (Tconv only)]. \* *p*<0.05

## 5.2.6 Production of anti-WT1 specific antibody

In order to determine the humoral immune responses to *E. coli*-LLO/WT1 vaccination, the presence of WT1 specific antibody was verified by Western Blot. Sera obtained from 6 mice, one week after the last vaccination with *E. coli*, showed no trace of WT1-antibody. The plasma of the 7/11 mice that received *E. coli*-WT1 vaccination were positive for WT1 antibodies and all of the 11 mice received *E. coli*-LLO/WT1 vaccination (Figure 44). Even though previous experiments (Karre, Ljunggren et al. 1986) had shown that the antigen presentation through *E. coli*-LLO vaccination mainly involved MHC class I pathway, this result demonstrated that some helper T-cell responses via MHC class II were also involved.

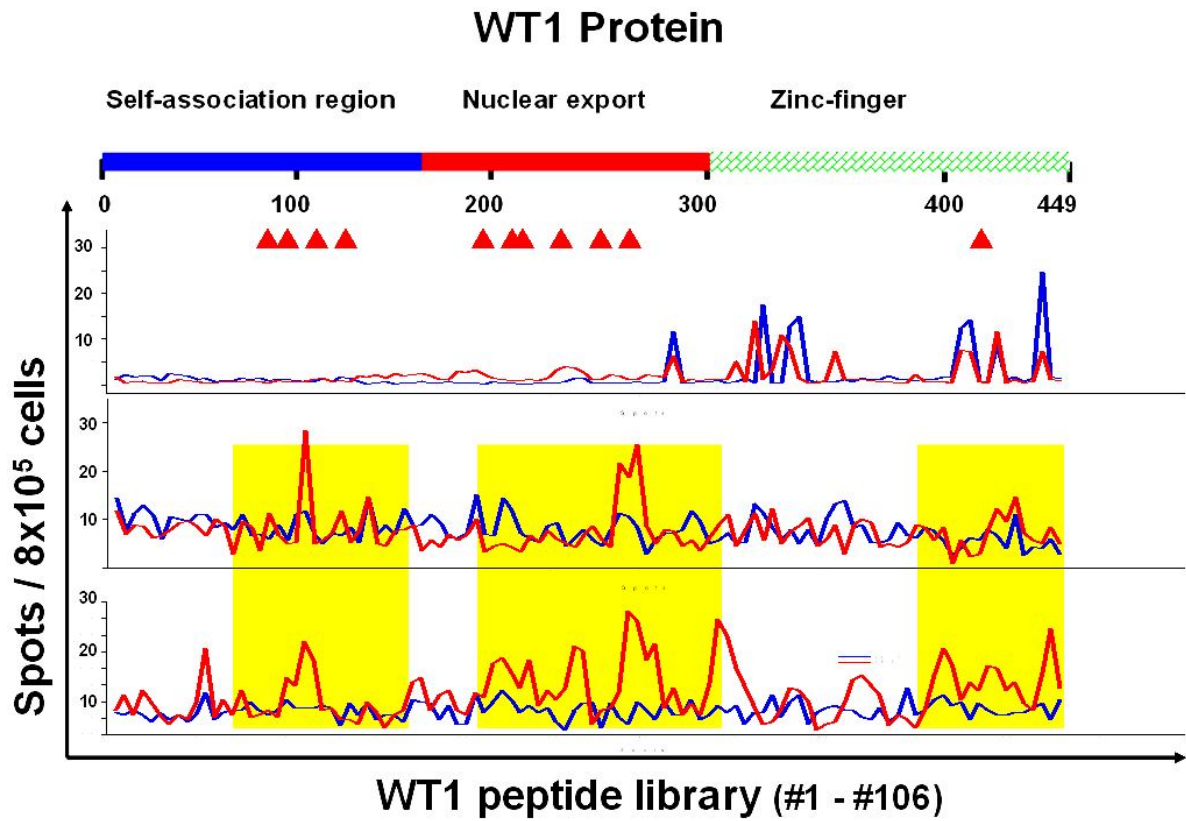


**Figure 44. Detection of WT1-specific antibodies by Western Blot.** Sera were obtained 1 week after the third immunisation. The presence of antibodies specific for WT1 was determined by Western Blot. Sera from *E. coli*-WT1 (7/11) and *E. coli*-LLO/WT1 (11/11) vaccinated mice showed the production of antibodies specific for WT1. Representative samples from 6 mice in each group are shown here. In contrast, no antibodies were detected in sera from *E. coli*- or *E. coli*-LLO-vaccinated mice (0/6, 2 representative samples).

## **5.2.7 Characterisation of immunodominant epitope following *E. coli*-LLO/WT1 vaccination**

### **5.2.7.1 Cytokine ELISpot screening of WT1 peptide library**

In order to characterise the immune dominant antigenic motifs involved in the anti-tumour effect of *E. coli*-LLO/WT1, a library of peptides (provided by Prof Stauss HJ, Royal Free hospital, London) covering the entire human WT1 protein (the human WT1 protein is 96% identical to the mouse protein) was screened (15-mer peptides, overlapping by 10-mer). Splenocytes extracted from 10 mice vaccinated with *E. coli*-LLO/WT1 were pooled and mixed with individual peptides in a 96-well plate. After twenty-four hours of stimulation, an ELISpot assay for interferon- $\gamma$  (IFN- $\gamma$ ) and IL-2 was carried out (Figure 45). This screening identified 12 different peptides (three 9-mer peptides and nine 15-mer peptides) capable of inducing significant IFN- $\gamma$  production, suggesting an involvement of these peptides in the anti-tumour effect (Figure 46, four peptides were shown).



**Figure 45. ELISpot screening of the WT1 peptide library.** Pooled splenocytes were harvested from mice immunised with fixed *E. coli*-LLO/WT1. The specific IFN- $\gamma$  (red) and IL-2 (blue) responses were measured by spots counting in ELISpot when stimulated with the whole panel of WT1 peptide library (total 106 15-mer peptides). Data represent 3 times of ELISpot screening. The selected 12 peptides are demonstrated by red closed triangles.

Peptide No.	Peptide Sequence	2 <sup>nd</sup> ELISpot			3 <sup>rd</sup> ELISpot		
<b>22</b>	85-99	28	32	24	19	13	14
<b>30</b>	117-131	18	12	8	12	8	14
<b>32</b>	125-139	15	18	31	14	11	19
<b>33</b>	129-143	25	7	43	12	20	16
<b>52</b>	205-219	10	6	19	11	5	24
<b>53</b>	209-223	7	19	20	10	18	16
<b>57</b>	225-239	22	20	23	25	26	5
<b>58</b>	229-243	19	23	13	12	24	15
<b>59</b>	233-247	25	19	42	10	12	24
<b>68</b>	269-283	11	13	17	18	17	17
<b>69</b>	273-287	6	8	19	11	5	17
<b>105</b>	417-431	15	12	26	20	14	14
<b>Medium</b>	-	3	4	2	2	1	4

**Table 9. IFN- $\gamma$  ELISpot responses in selected peptides.** IFN- $\gamma$  responses to 12 selected peptides and medium control from the 2<sup>nd</sup> and 3<sup>rd</sup> ELISpot screening were presented as spot numbers per well.

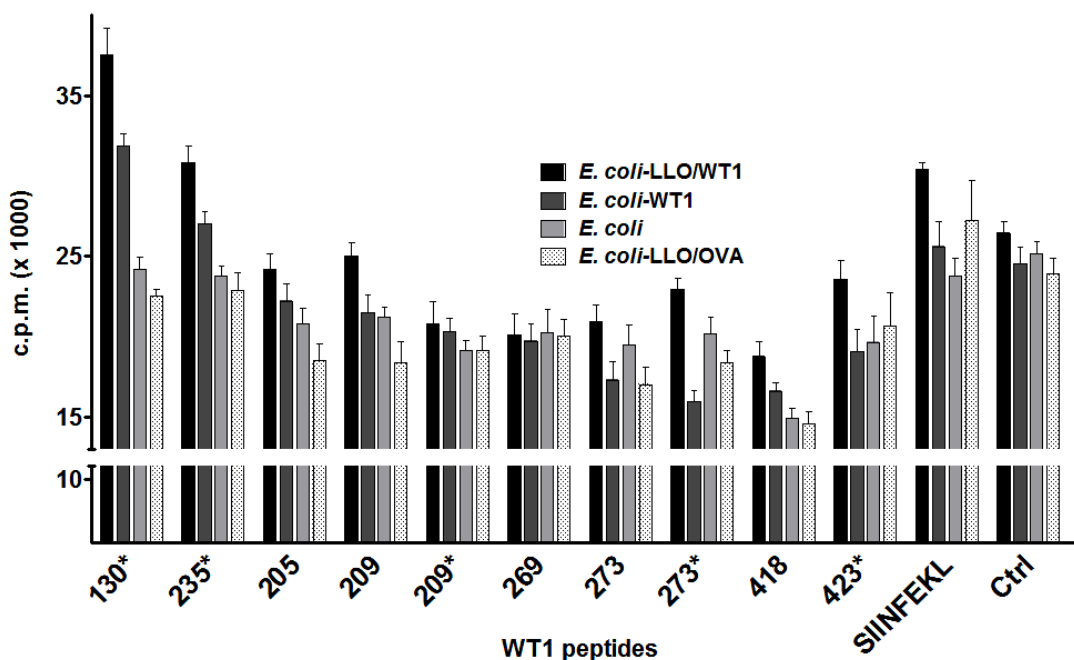
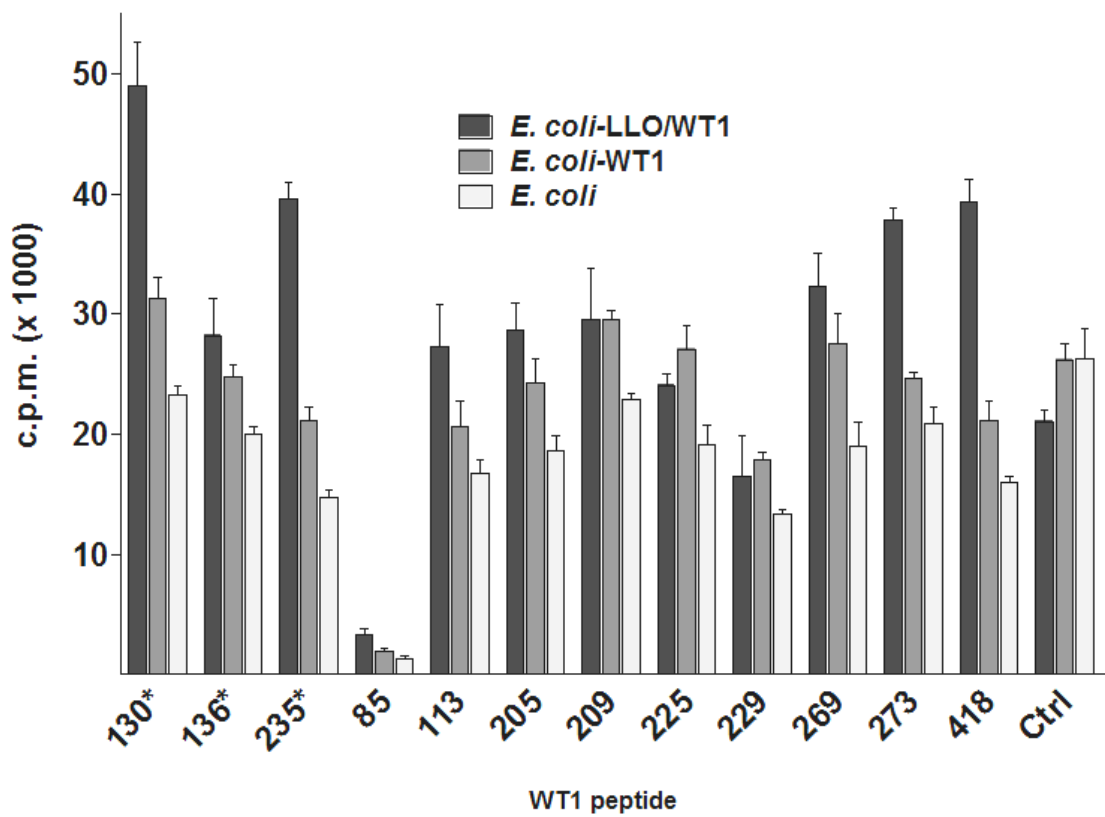
### 5.2.7.2 Lymphocyte proliferation assay (LPA)

#### LPA on pooled splenocytes

Since the numbers of spot obtained from ELISpot screenings were generally low (between 15-30 spots/well), the peptide-specific cytokine responses needed to be validated by peptide-induced lymphocyte proliferation. Subsequently, these 12 peptides selected were used to stimulate *in vitro* the splenocytes obtained from animals vaccinated with *E. coli*-LLO/WT1 or *E. coli*-WT1 and splenocytes from *E.*



*coli*-vaccinated animals were used as controls (Figure 46, the number indicated the starting sequence of the peptide and peptides marked with \* are 9-mer, otherwise are 15-mer). In preliminary experiments, no significant response was observed when stimulated with 10  $\mu$ M or 20  $\mu$ M peptide without IL-2. Therefore, subsequent experiments regarding proliferation assays were all carried out with peptide (20  $\mu$ M) in the presence of IL-2 co-stimulation. In consideration of the effect of DMSO within the peptide solution, a DMSO control group was also included in addition to medium-only control. *p*WT 130\* had the consistently best responses among all the peptides. In addition, peptide *p*WT 235\*, *p*WT 269, *p*WT 273, *p*WT 418 also gave significant proliferative responses compared to *E. coli* group. *p*WT 113, *p*WT 205 and *p*WT 209 had moderate responses compared to *E. coli* group and non-peptide medium control. In further experiments examining the antigenic epitope related to *E. coli*-LLO/WT1 vaccination, we re-tested all the peptides mentioned above. In addition, the overlapping 9-mer peptides between *p*WT 205/209 and *p*WT 269/273 (*p*WT 209\* and *p*WT 273\*), and the 9-mer peptide within *p*WT 418, were synthesised to be included in the panel of proliferation assay. In this experiment, *p*WT 130 and *p*WT 235 induced proliferations consistently compared to either *E. coli*-vaccination group or non-peptide control. The 9-mer peptide (*p*WT<sub>423-431</sub>) showed improved responses compared to 15-mer *p*WT<sub>418-432</sub>, whereas the selected *p*WT 209\* and *p*WT 273\* did not demonstrate any better results than the original separated 15-mer peptides (Figure 47).

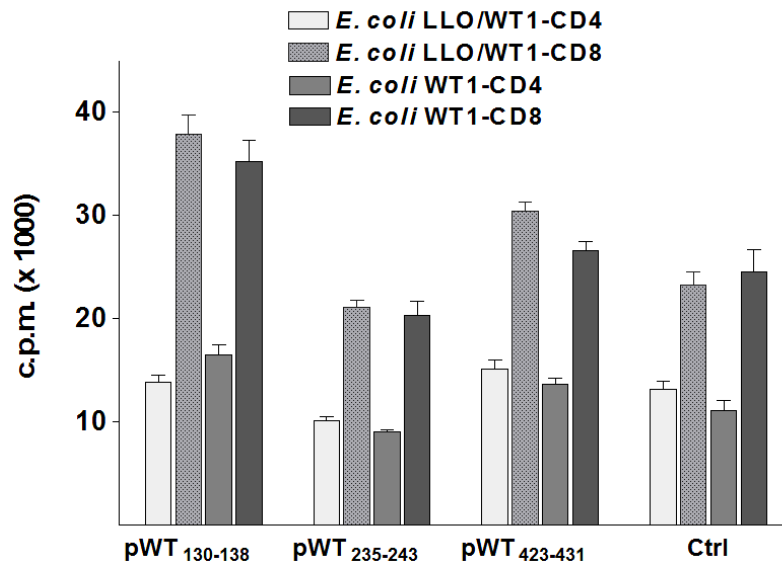


**Figures 46 and 47. Lymphocyte proliferative responses to selected WT1 peptides.** In culture plates stimulated with peptide 20  $\mu$ M in the presence of, overall proliferative response was significantly better in lymphocytes from *E. coli-LLO/WT1*-vaccinated mice than from *E. coli* or *E. coli-WT1*-vaccinated mice. When looking into the results in *E. coli-LLO/WT1*-vaccinated mice and compared to the other 2 groups, pWT130\*, pWT 235\*, pWT 269, pWT 273, and pWT 423 elicited better proliferation than other peptides. The results were

consistent with previous data (not shown) that pWT 130\* gave the consistent proliferative response *in vitro*. In another experiment testing the lymphocyte proliferation, three 9-mer peptides, pWT 209\*, pWT 273\*, and pWT 423\* were included in the panel of examining peptides. The OVA<sub>257-264</sub> epitope, SIINFEKL, which is a well-documented motif, was also included in the tested peptide. Another group of mice vaccinated with *E. coli*-LLO/OVA was included for another control.

### LPA on purified CD4 and CD8 cells

To determine the cell-type stimulated by the various peptides, we purified CD4<sup>+</sup> and CD8<sup>+</sup> cells before peptide stimulation. Subsequent proliferation testing was performed in the presence of IL-2 co-stimulation and 20 μM peptide (Here I chose three 9-mer peptides which have the best proliferative responses). All the peptides led to significant CD8<sup>+</sup> cells proliferation rather than CD4<sup>+</sup> cells. pWT 130 had shown the consistent significant responses compared to other peptides (Figure 48).



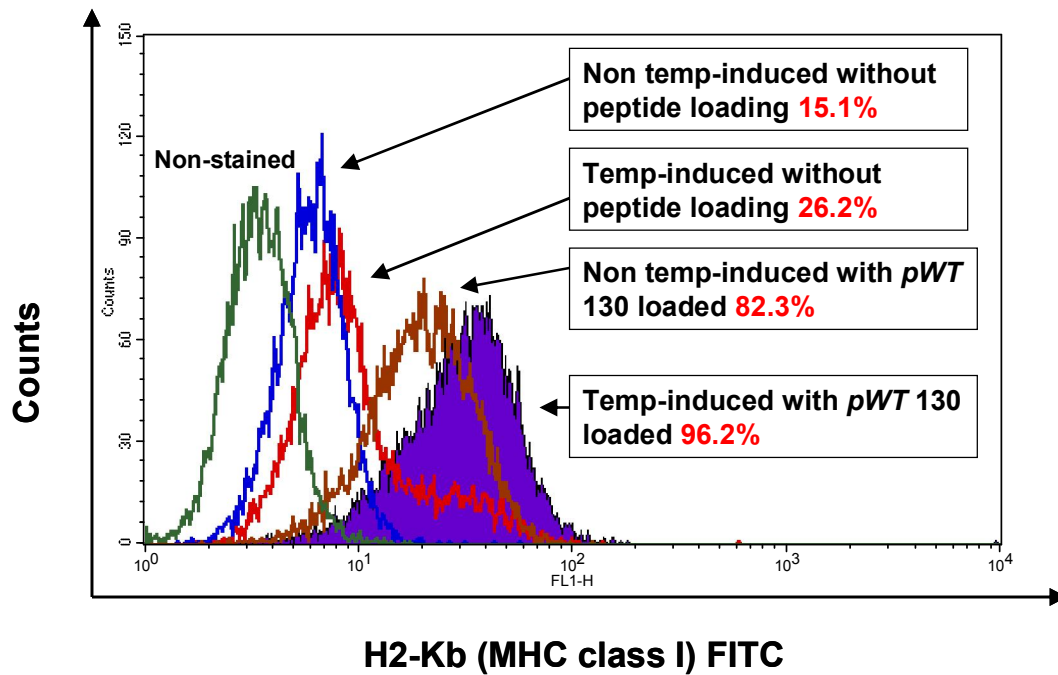
**Figure 48. CD4<sup>+</sup> or CD8<sup>+</sup> lymphocyte proliferative responses to WT1 peptides.** CD4<sup>+</sup> or CD8<sup>+</sup> cells from immunised mice were purified by anti-CD4 or anti-CD8 magnetic microbeads (MACS, Miltenyi Biotech, Germany) and stimulated by WT1 peptide 20 μM *in vitro* in the presence of IL- 2 and irradiated syngeneic splenocytes as feeders. Medium

without peptide was used as control group. The overall T-cell subset proliferation determined by <sup>3</sup>H-Thymidine incorporation is prominent in CD8+ cells.

### **5.2.7.3 Cytotoxicity assay**

#### *MHC class I expression in peptide-loaded RMA-S cells*

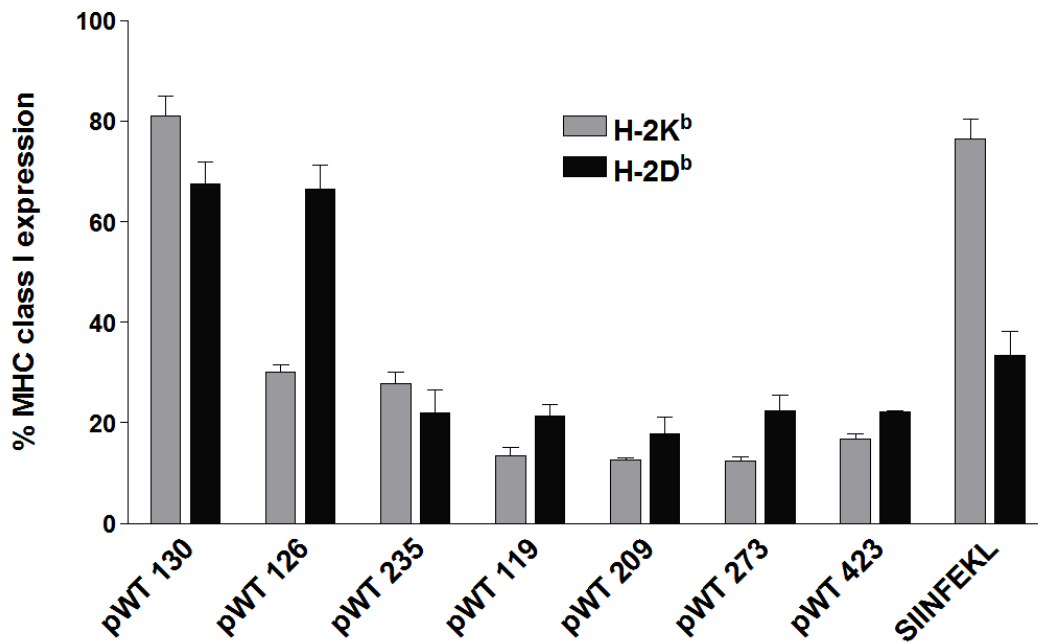
RMA-S cells were used in MHC binding assays to determine the class I binding efficiency of synthetic WT1 peptides. In this experiment, I used pWT 130 for testing MHC class I expression in RMA-S cells. RMA-S cells express 5% of the level of H2-K<sup>b</sup>/D<sup>b</sup> (Rammensee, Bachmann et al. 1999). When RMA-S cells are cultured at 26°C, this leads to increased expression of empty class I molecules. These empty class I molecules are unstable and fragile when RMA-S cells are shifted to 37°C but can be stabilised when class I binding peptides are present in the culture medium. The MHC class I expression (H2-K<sup>b</sup>) after pWT130 loading to temperature-induced RMA-S cells can achieve 96.2% (Figure 49) indicating the stable expression of MHC-I and ideal for the target in CTL assay.



**Figure 49. Temperature-induced MHC class I expression in RMA-S.** The highest expression of MHC class I in RMA-S cells was shown upon specific temperature (26°C)-induction and loaded with an immunogenic peptide (pWT 130).

### Peptide binding stability

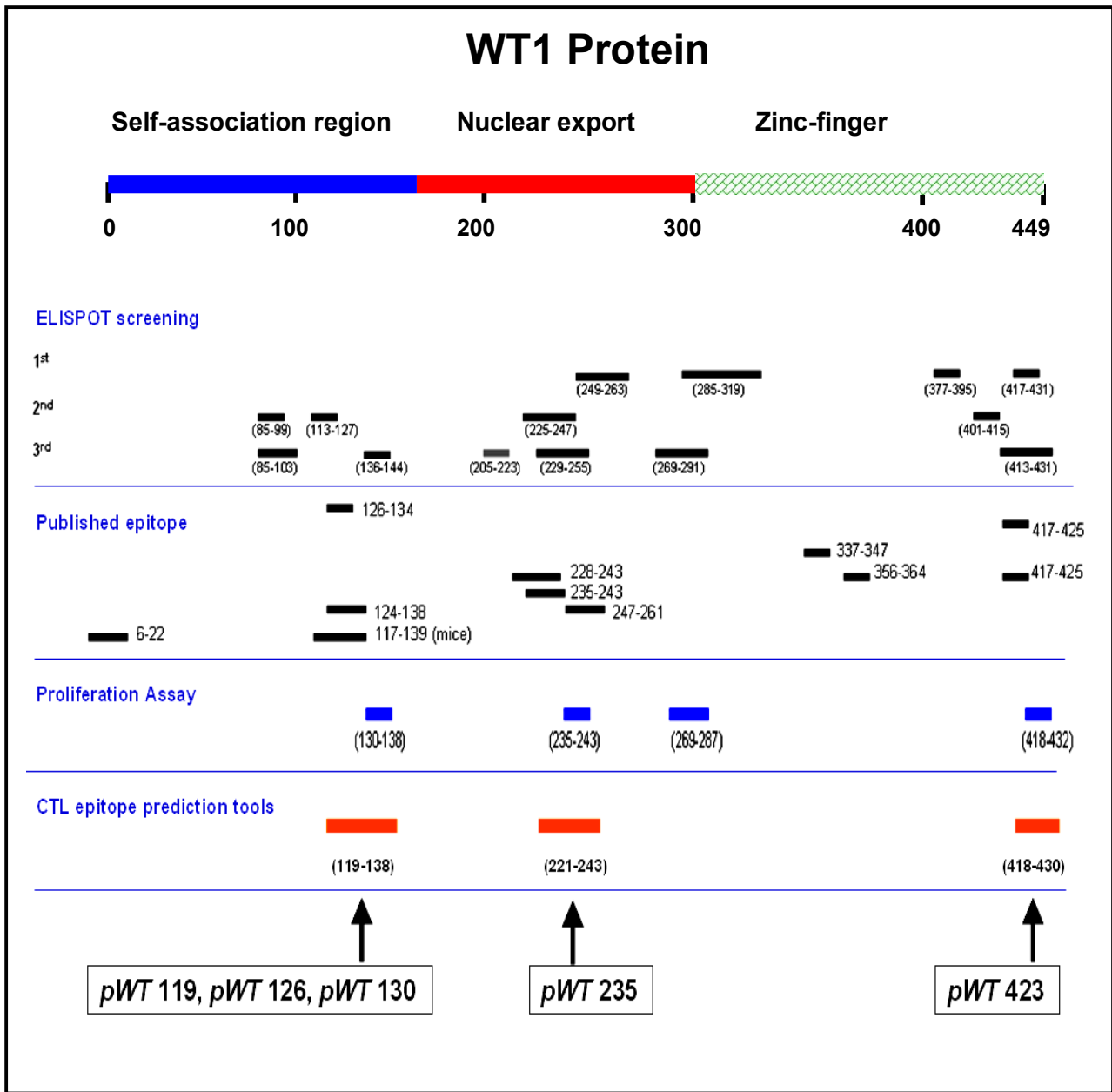
Selected 9-mer WT1 peptides/motifs to stabilise MHC class I (H-2<sup>b</sup>) expression were tested. Results shown in Figure 50 show that few chosen WT1 peptides stably bind to MHC class I molecules. In comparison to SIINFEKL, OVA<sub>257-264</sub>, which is a well-documented CTL antigenic epitope of OVA, only pWT 130 (both H2-K<sup>b</sup> and H2-D<sup>b</sup>) and pWT 126 (predominantly H2-D<sup>b</sup>) showed significant superior binding stability to the MHC class I expressed by RMA-S.



**Figure 50. MHC class I binding stability of WT1 peptides.** Temperature-induced RMA-S cells were incubated with each WT1 peptide in 10  $\mu$ M concentration. Cells were cultured at 37°C for 2 hours, labeled with anti-D<sup>b</sup> or anti-K<sup>b</sup> mAb and then analysed by flow cytometry to determine the level of MHC class I expression. Results from a representative experiment are shown where the mean fluorescence intensity is represented against each WT1 peptide. The OVA<sub>257-264</sub> epitope, SIINFEKL, was used as a positive control.

To sum up from all the results obtained from ELISpot screening, lymphocyte proliferation assay, and the peptide binding stability on MHC class-I, I chose three 9-mer peptides, pWT 130, pWT 235, and pWT 423, to be tested in subsequent CTL assay (Figure 51). In reviewing all previous published CTL or HTL epitopes related to WT1 peptide, pWT 126 has been the most commonly discussed and applied. In addition, 9-mer pWT 235 has been applied in phase I/II clinical trial for cancer immunotherapy and some pilot studies in leukaemic patients. Several websites are also available to provide the prediction algorithm for CTL or HTL epitopes and I hereby selected two 9-mer peptides with highest estimated binding score to H2-D<sup>b</sup> in 2 separate websites: pWT 119 from <http://bio.dfci.harvard.edu/Tools/rankpep.html>

and pWT 126 from <http://www.uni-tuebingen.de/uni/kxi/> and SYFPEITEI <http://www.syfpeithi.de/home.htm> (Edelman and Tacket 1990). pWT 209 and pWT 273 that gave moderate responses from lymphocyte proliferation assay were not selected into the panel of CTL assay because of their poor MHC class-I binding ability and relative low prediction MHC binding score. Figure 52 shows all the selection strategies I have used and the final choice of five candidate epitopes to be further examined in subsequent CTL assay. In addition, pWT130, which consistently provided the best results in previous experiments, was considered for the generation of peptide-MHC tetramer/pentamer. However, this strategy was abandoned as this MHC-pentamer failed to be manufactured by *ProImmune Ltd* (Oxford, U.K.).



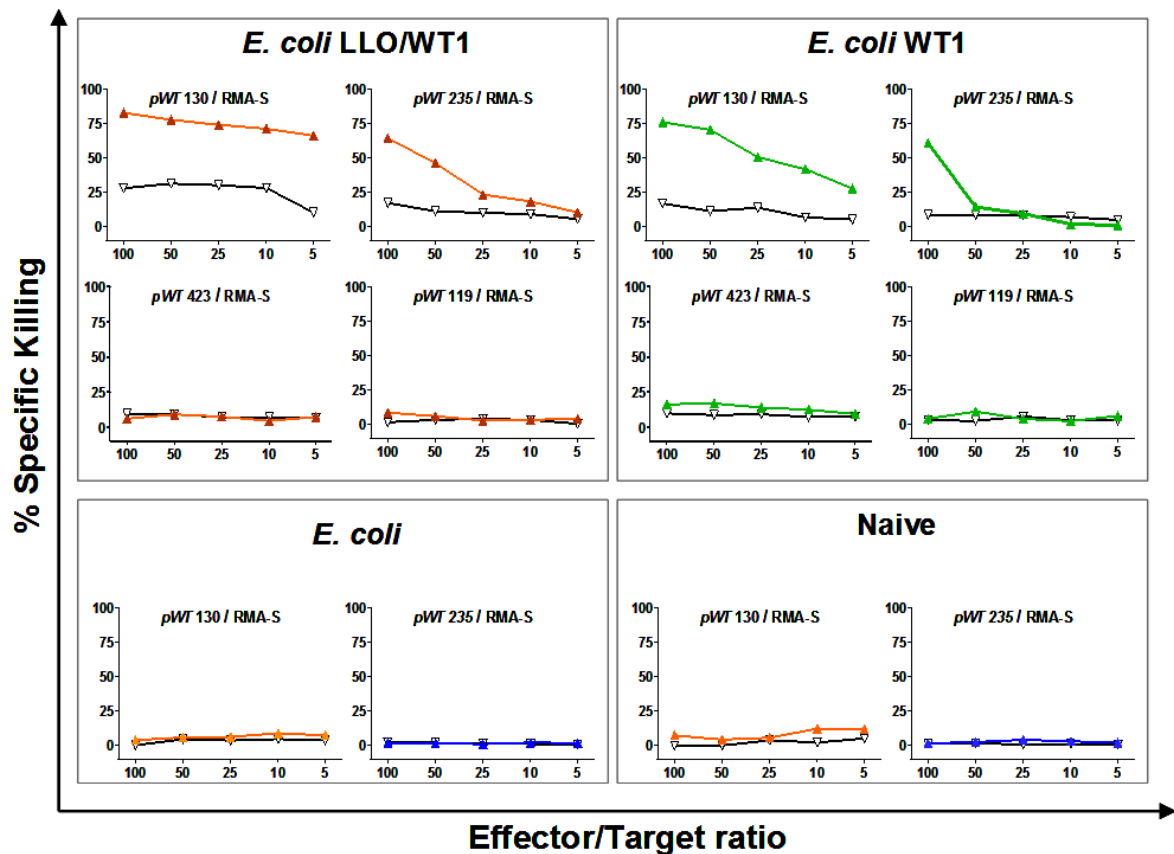
**Figure 51. Schematic representation of the strategies to select the candidates of WT1 immunodominant epitopes.** Five peptides (9-mer) were chosen based on the data of ELISpot screening, published WT1 CTL epitope (for either *in vitro* study or *in vivo* application), *in vitro* lymphocyte proliferation by peptide stimulation, and two CTL epitope prediction websites. These peptides were considered to be the potential antigenic epitope recognised by CD8+ T-cell through *E. coli*-LLO/WT1 vaccination and they will be assessed by *in vitro* CTL killing ( $^{51}\text{Cr}$  releasing assay).



### Cytotoxicity ( $^{51}\text{Cr}$ release) assay

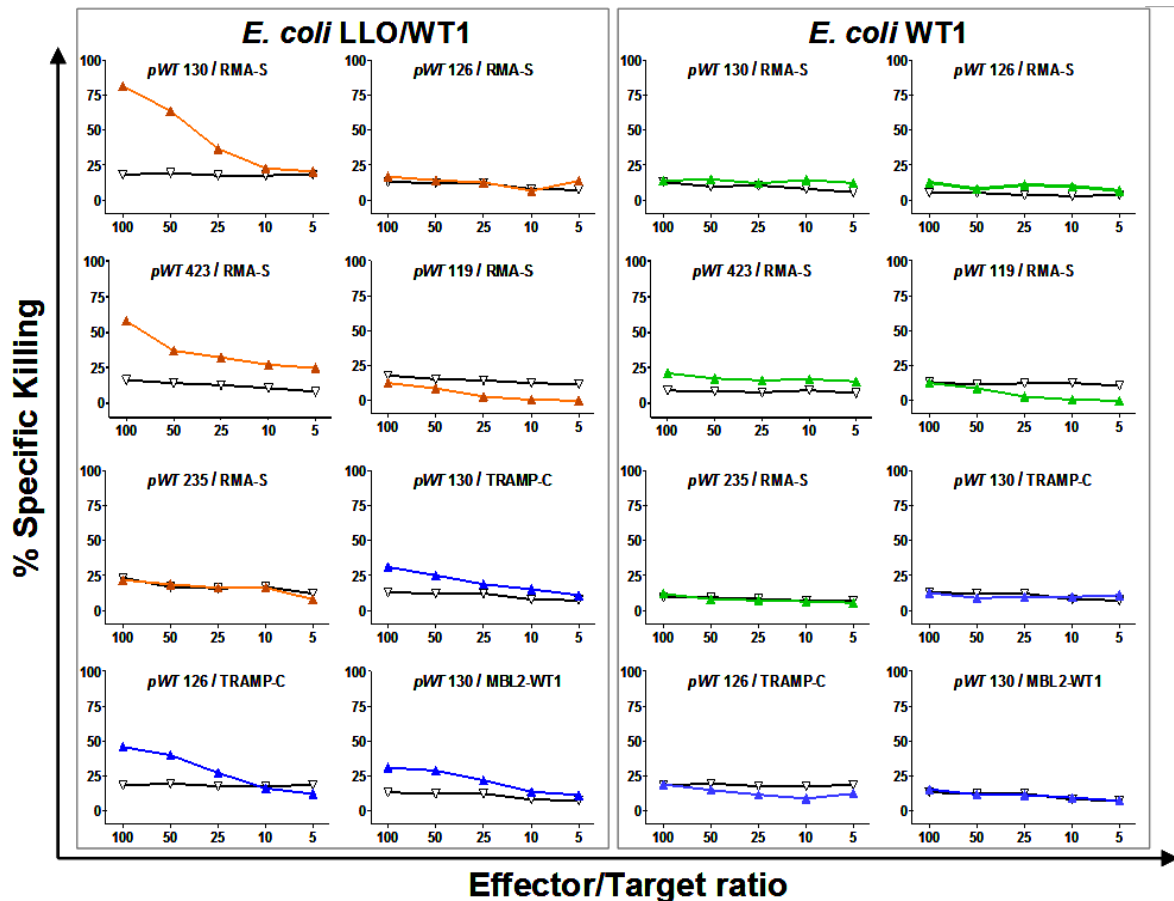
To characterise the presence of the antigen-specific cytotoxic responses and determine the CTL epitope related to *E. coli*-LLO/WT1 vaccination, splenocytes from vaccinated mice were re-stimulated *in vitro* with WT1 peptide-loaded LPS-activated splenocytes and CTL activity was measured 6 days later using WT1 peptide-loaded RMA-S and WT1-expressing cells, TRAMP-C and MBL2-WT1 as targets. When peptide-loaded RMA-S cells were used as target, irrelevant peptide (SIINFEKL)-loaded RMA-S cells were used as control target cells. The results are summarised in Figures 52-54.

Splenocytes from vaccinated mice were re-stimulated *in vitro* with WT1 peptide-loaded LPS-activated splenocytes and CTL activity was measured 6 days later using WT1 peptide-loaded RMA-S. RMA-S loaded with irrelevant peptide (SIINFEKL) was used as control target. A significant cytotoxicity killing to pWT 130-loaded RMA-S was obtained in the *E. coli*-LLO/WT1 and *E. coli*-WT1-vaccinated group compared to the naïve or *E. coli*-vaccinated group. A moderate cytotoxicity killing to pWT 235-loaded RMA-S at the highest E/T ratio was observed in the *E. coli*-LLO/WT1 and *E. coli*-WT1-vaccinated groups compared to naïve or *E. coli*-vaccinated groups. Especially, the yield of specific CTLs from pWT 130 stimulation was higher in *E. coli*-LLO/WT1 group than *E. coli*-WT1 because of persistent killing from high to low E/T ratio. There was no significant killing to pWT 119 or pWT 423-loaded RMA-S in all groups (Figure 52).



**Figure 52. *E. coli*-LLO/WT1 vaccination generates specific CTL responses (1).** C57BL/6 mice received 3 s.c. injections of bacteria at one week intervals. Splenocytes were harvested one week later and re-stimulated *in vitro* with LPS-activated spleen cells loaded with five selected WT1 peptides. Cytotoxicity was assayed 6 days later by  $^{51}\text{Cr}$  release assay against WT1 peptide or irrelevant -loaded RMA-S cells. Specific  $^{51}\text{Cr}$  release was measured as described in the methods. Results from individual groups are plotted against the E:T ratio.

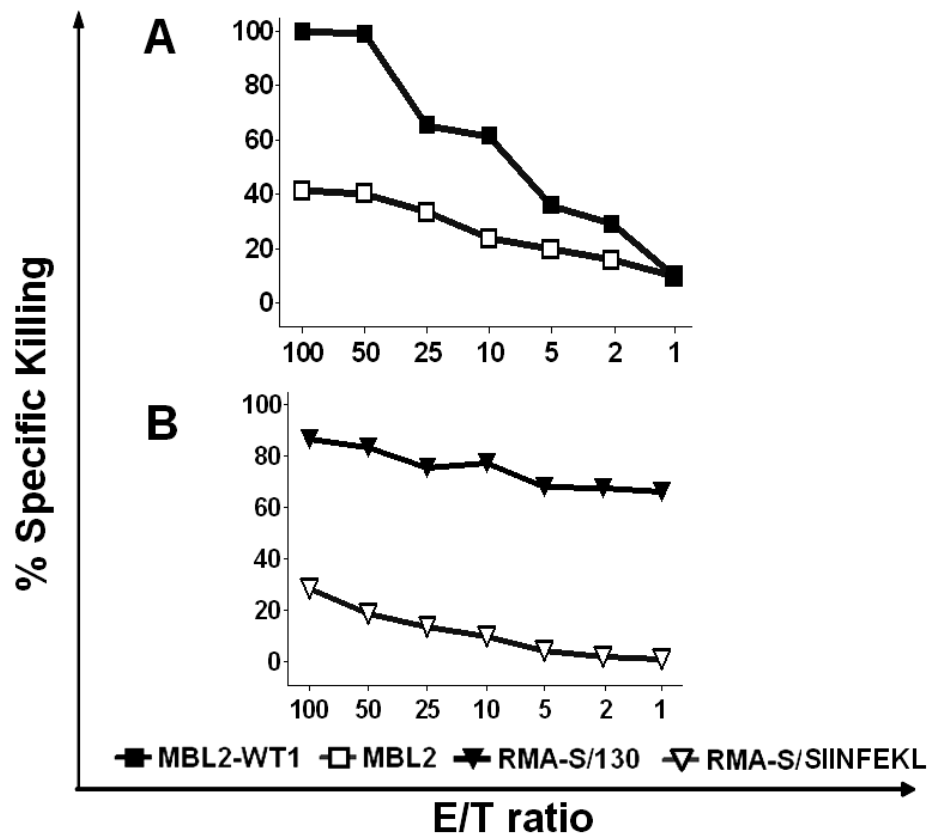
Splenocytes from vaccinated mice were re-stimulated *in vitro* with WT1 peptide-loaded LPS-activated splenocytes and CTL activity was measured 6 days later using WT1 peptide-loaded RMA-S and tumour cells (TRAMP-C and MBL2-WT1) as target cells. RMA-S loaded with irrelevant peptide (SIINFEKL) and MBL2 were used as control target. A significant cytotoxicity killing to pWT 130-loaded RMA-S was obtained in *E. coli* LLO/WT1 but not in *E. coli* WT1-vaccinated group.



**Figure 53. *E. coli*-LLO/WT1 vaccination generates specific CTL responses (2).** C57BL/6 mice received 3 s.c. injections of bacteria at one week intervals. Splenocytes were harvested one week later and re-stimulated *in vitro* with LPS-activated spleen cells loaded with five selected WT1 peptides. Cytotoxicity was assayed 6 days later by  $^{51}\text{Cr}$  release assay against WT1 peptide-loaded RMA-S cells or WT1-expressing tumour cells (TRAMP-C and MBL2-WT1 cells). Irrelevant peptide (SIINFEKL)-loaded RMA-S and parental cells MBL2 were used as control target. Specific  $^{51}\text{Cr}$  release was measured as described in the methods. Results from individual mice are plotted against the E:T ratio.

In the vaccination protocol (Figure 41 in Section 5.2.3.2), the mice in the *E. coli*-LLO/WT1 group survived the challenge with MBL2-WT1 and were considered as “cured”. A second MBL2-WT1 tumour challenge was performed to the tumour-free mice 40 days after the first tumour challenge. There was no tumour re-growth in all the re-challenged mice 30 days after second challenge. Splenocytes from these mice

were collected, *in vitro* stimulated, and used as effector cells. A significant cytotoxic killing was demonstrated to pWT 130-loaded RMA-S and MBL2-WT1 compared to control targets (>80% specific killing at the highest effector to target ratio), suggesting the significant presence of antigen-specific CTLs (Figure 54).



**Figure 54. Specific CTLs obtained from tumour-resistant mice.** The mice received 3 immunisations of *E. coli*-LLO/WT1 and were challenged with MBL2-WT1 ( $5 \times 10^6$  cells/mouse) one week after the last injection. Tumour-free mice were re-challenged (MBL2-WT1  $5 \times 10^6$  cells/mouse) 40 days after the initial challenge. All the mice were protected from the tumour re-challenge without tumour re-growth and the total splenocytes were harvested and stimulated with (A) irradiated MBL2-WT1 cells or (B) LPS-activated lymphocytes loaded with pWT130 and then used as effector cells. These cells demonstrated 80-100% specific cytotoxicity of target cells (A) MBL-2-WT1 or (B) RMA-S/130 at the highest effector/target ratio attributed to the specific CTLs.

In summary, pWT 130 appears to be the major antigenic epitope that stimulates T cell proliferation (CD8<sup>+</sup>) and mediates *E. coli*-LLO/WT1 anti-tumour effect. pWT 130 gave consistent results compared to other testing peptides. However, the role of pWT 126, pWT 235, and pWT 423 involved in this vaccine are not conclusive as they had inconsistent target killing abilities in one of these experiments (pWT 126 to TRAMP-C, pWT 423 to RMA-S, and pWT 235 to RMA-S) and also had modest stimulatory effect to lymphocyte proliferation.

### 5.3 CONCLUSION

The data demonstrated that an *E. coli*-LLO vaccine, which expresses an oncofetal tumour antigen, WT1, is capable of inducing a strong anti-tumour effect against implanted WT1-expressing tumours *in vivo*. The mechanisms were mainly attributed to its ability to induce specific CTLs and inhibit the suppressive function of Foxp3<sup>+</sup> Treg, as we have seen in the model antigen system. Furthermore, we have characterised the immunodominant epitope (pWT<sub>130-138</sub>, NPYLPSC<sub>L</sub>) involved in this effect. Even though the MHC multimeric assay is technically not feasible to detect the antigen-specific CTLs, the presence of CTLs against this peptide was demonstrated using a <sup>51</sup>Cr-release assay.

# CHAPTER 6: THE ADJUVANT ROLE OF *E. COLI*-LLO BACTERIA IN PEPTIDE VACCINE

## 6.1 INTRODUCTION

Vaccination works by manipulating the immune response through selecting, activating and expanding the memory of B and T cells. To determine the magnitude and quality of immune response, suitable vaccine adjuvants are required; therefore, much effort is going into finding new, effective and non-toxic adjuvant formulations focussed on the activation of key immune targets for inducing a long-term, potent and safe immune response. Vaccine adjuvants can act in several, non-mutually exclusive ways to augment the adaptive immune response and to generate effective immunological memory through contacting with APC, such as DCs. Thereby adjuvants can affect the migration, maturation, antigen presentation, and expression of costimulatory molecules by DCs, and these events in turn improve the responses to antigen of T and B cells. Adjuvants can also affect the nature of CD4<sup>+</sup> T helper (Th), CD8<sup>+</sup> T cell, and B cell responses, with some adjuvants promoting Th1-related responses and others preferentially inducing Th2-biased effects. Furthermore, some adjuvants enhance cross-presentation by DCs of MHC I-restricted antigens to CD8<sup>+</sup> T cells. Adjuvants may also act directly in improving the effector cells proliferation and/or conversion into memory cells that are essential for the success of vaccines.

Adjuvants are generally immunogenic, mainly function to target associated antigens into APCs and create a depot for sustained immune stimulation. Immunostimulatory adjuvants are predominantly derived from microbials and often represent PAMPs. A practical categorisation of different types of immunostimulatory

adjuvants has been proposed by Edelman and Tackett (Cox and Coulter 1997). Three general types are proposed: adjuvants *per se*; carriers; and vehicles. The adjuvant *per se* includes aluminium salts, saponin, muramyl di- and tripeptides, monophosphoryl lipid A, Bordetella pertussis, cytokines, and many others. The carriers, which mainly provide T cell help, include bacterial toxoids, fatty acids, and living vectors. The vehicle category includes mineral oil emulsions (e.g. incomplete Freund's adjuvant), biodegradable oil emulsions (e.g. emulsions containing peanut oil, squalene, or squalane), non-ionic block copolymer surfactants, liposomes, and biodegradable polymer microspheres. Another categorisation of adjuvants is based on five potential modes of adjuvant action: immunomodulation (modification of cytokine networks); presentation (maintenance of antigen conformation); CTL induction; targeting; and depot generation (McKee, Munks et al. 2007).

In some studies, delivery systems and immunostimulatory agents have been combined to prepare adjuvant delivery systems, which are designed for more effective delivery of the immunostimulatory adjuvant into APC (Wack, Baudner et al. 2008). A coupling of immunopotentiator on a delivery device may prolong its residence or target it to more relevant antigen presenting cells. O'Hagan et al showed that decoration of CpG motifs (signal 2 facilitators) onto PLG particles (Ag delivery devices) synergistically improves immunopotentiator functions and concomitantly reduces systemic side effects (Warger, Osterloh et al. 2006). Recent progress in the knowledge of innate immunity is beginning to yield insight into the initiation of immune responses and the ways in which immunostimulatory adjuvants may enhance this process. However, a rational approach to the development of new and more effective vaccine adjuvants will require much further work to better define the mechanisms of action of existing adjuvants.

Many of the vaccines currently used in humans contain adjuvants that are intrinsic to the immunogen. For example, vaccines that contain attenuated live or heat-killed viruses or bacteria include components that can engage TLRs. These components therefore act as natural adjuvants because TLR signalling has many of the effects on DC antigen presentation that one would wish for an adjuvant: improvement in antigen presentation and increases in costimulatory molecules and cytokine production, leading usually to improved Th1-related responses. Importantly, incorporation of TLR as an adjuvant in vaccination can reverse the Treg suppression and potentiate the adaptive immune responses counterbalanced by Treg (Conroy, Marshall et al. 2008). However, a problem with TLR agonists that has not been fully appreciated is that they can generate suppressive as well as inflammatory responses in innate immune cells and can promote the induction of regulatory as well as effector T cells (Smith and Cerundolo 2001). Peptide vaccines combined with IFA were insufficiently immunogenic and did not elicit robust anti-tumour immune responses in the absence of exogenous cytokines (Gupta, Relyveld et al. 1993). Although IFA stands as one of the most successful adjuvants, both for animals and humans, its potential toxicity has been proposed (Heit, Gebhardt et al. 2008).

The primary goal in the development of vaccine adjuvant is to induce potent and long-lasting immune effectors and memory cells. However, the primary immune responses raised by vaccination are also characterised by the activation of counter-regulatory mechanisms, which are necessary to prevent excess T cell expansion or maintenance of autoreactive T cells. Treg seem particularly to be adept at controlling immune responses to self antigens as well as pathogens. In particular, antigen-induced Treg, instead of natural Treg, can recognise foreign antigens and develop during prolonged antigen exposure such as immunisation with purified antigens with



conventional adjuvants (Heit, Gebhardt et al. 2008). This is a drawback when attempting to create a sustained antigen stimulation depot by an adjuvant. Because of their potent suppressive activity on immune responses *in vivo*, temporary down-modulation of Treg by the vaccine adjuvant has been suggested as an attractive target to improve the efficacy of vaccines. Heit et al have showed that depletion of Treg can lead to generation of long-living memory T cells (Peng, Guo et al. 2005). Moreover, the positive adjuvant effect of TLR ligands has been partially linked to the Treg compartment, either by direct reversion of Treg function (Pasare and Medzhitov 2003) or by TLR-induced cytokines (such as IL-6, by TLRs upon recognition of microbial products), which make responding T cells refractory to suppression by Treg (Dannull, Su et al. 2005).

## **6.2 RATIONALE AND AIM**

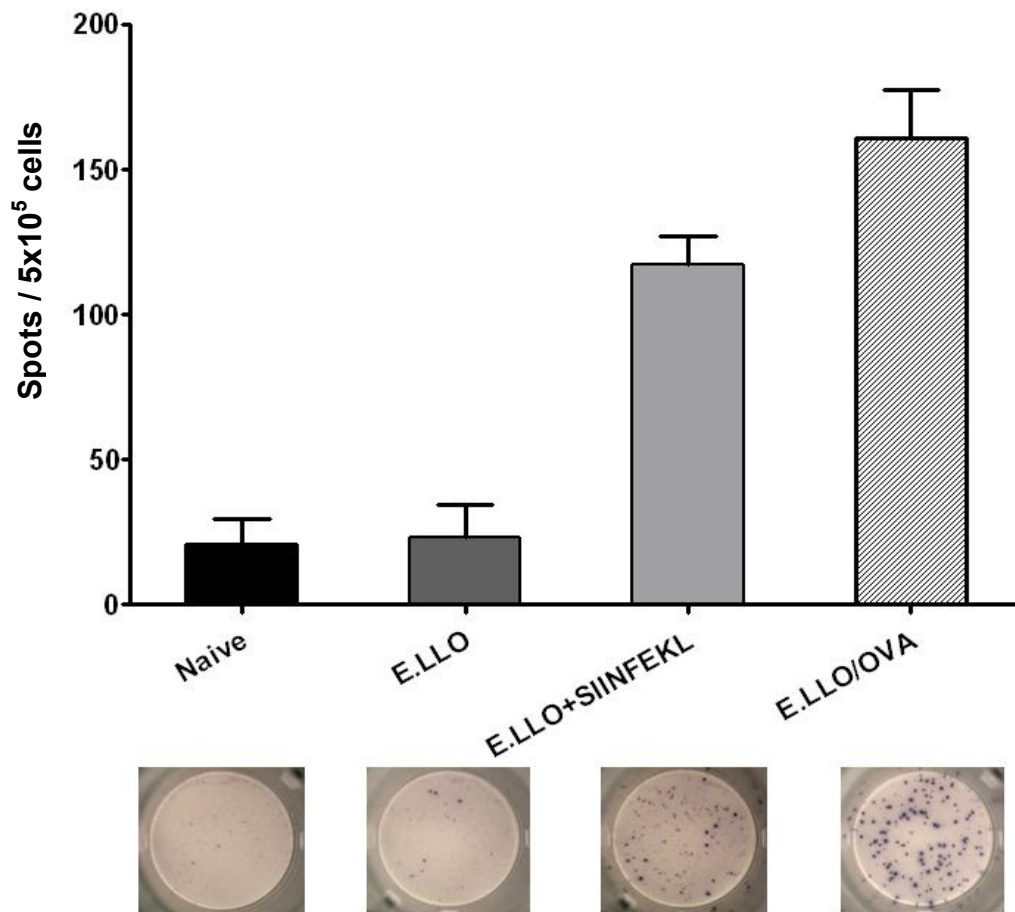
In Chapter 3 and Chapter 5, I have demonstrated that *E. coli*-LLO vaccination can reduce the Treg suppression on Tconv proliferation *in vitro* (Figures 17 and 22) but without emergence of specific CTLs. The aim of this study is to determine whether the vehicle, *E. coli*-LLO, can be simply exploited as an adjuvant to peptide vaccination. If successful this approach could be extended more easily to human study.

In this part, I intended to compare the immunological responses of peptide vaccination with *E. coli*-LLO adjuvant to antigen expression within *E. coli*-LLO (*E. coli*-LLO/OVA and *E. coli*-LLO/WT1).

## 6.3 RESULTS

### 6.3.1 Cytokine ELISpot analysis

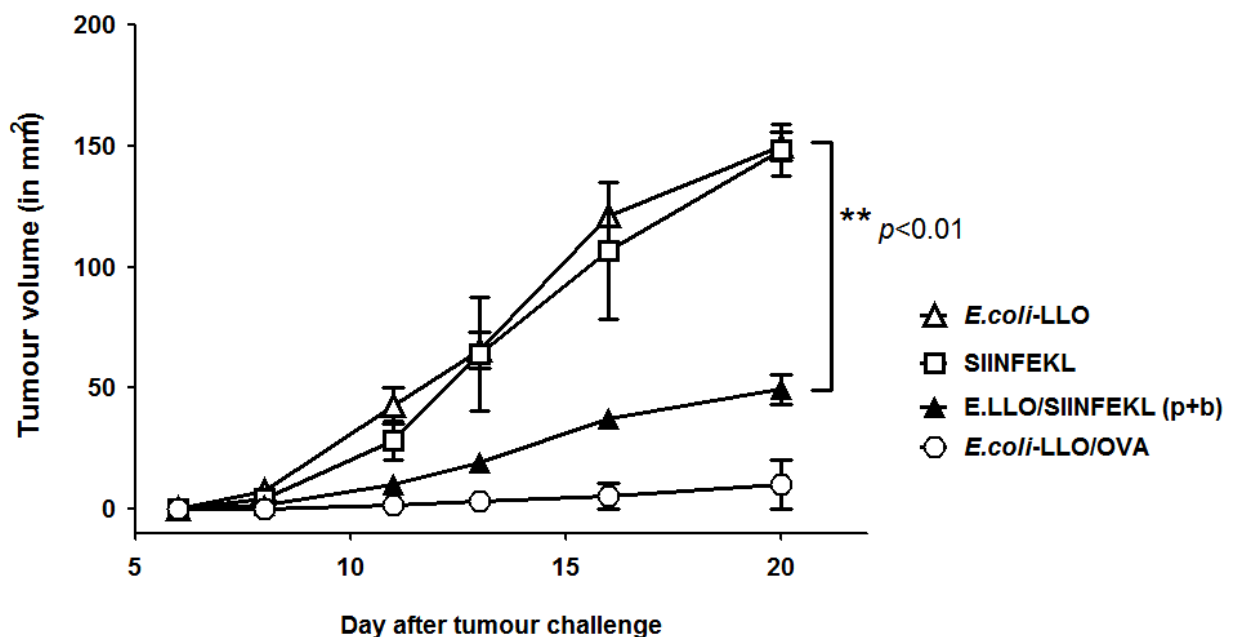
In the mice vaccinated with *E. coli*-LLO with OVA<sub>257-264</sub>, there was a significantly better response in the IFN- $\gamma$  ELISpot analysis compared to *E. coli* and *E. coli*-LLO, even though this response was not as good as OVA-expressing *E. coli*-LLO (Figure 55).



**Figure 55. IFN- $\gamma$  ELISpot response.** Mice received two s.c. immunisations of *E. coli*-LLO, *E. coli*-LLO+SIINFEKL, or *E. coli*-LLO/OVA, (at  $10^8$  bacteria /mouse or 100  $\mu$ g peptide/mouse) at one week intervals. Splenocytes were collected one week after the boost injection and incubated overnight with the SIINFEKL peptide, and ELISpot assay was used to measure IFN- $\gamma$  secretion. Results are expressed as mean  $\pm$  SD. (\*\*  $p < 0.01$ , *E. coli*-LLO+SIINFEKL v.s. *E. coli*-LLO)

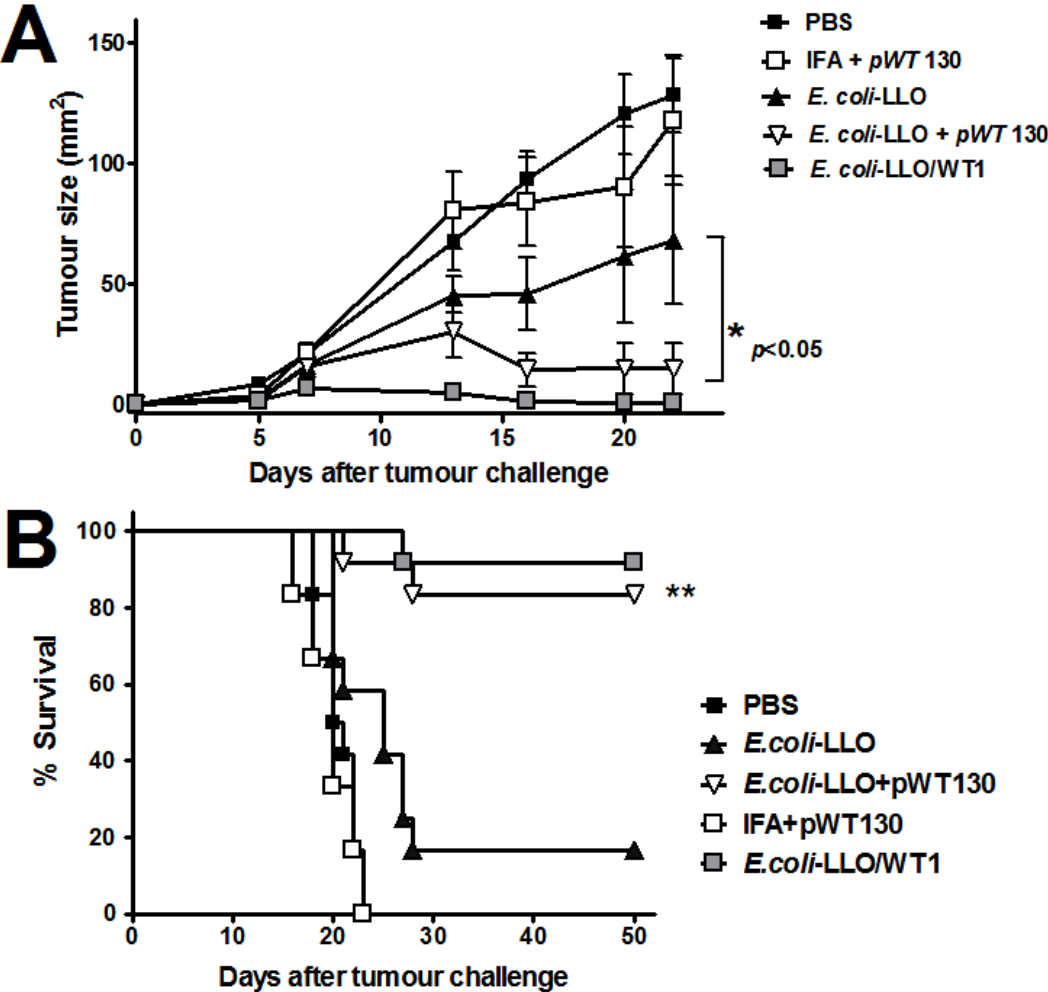
### 6.3.2 Tumour protection effects with peptide vaccination with *E. coli*-LLO

To determine whether there is a requirement to deliver the antigen within the bacterial vaccine or whether it can be administered separately, I compared the protection obtained with vaccination with *E. coli*-LLO/OVA to that obtained with a vaccination with *E. coli*-LLO with OVA<sub>257-264</sub> (SIINFEKL). Regarding the anti-tumour effect *in vivo*, the mice were immunised twice with *E. coli*-LLO, SIINFEKL, *E. coli*-LLO with SIINFEKL, OVA plasmid prime and *E. coli*-LLO/SIINFEKL boost, or *E. coli*-LLO/OVA. The mice immunised with *E. coli*-LLO with SIINFEKL had a significant tumour protection effect similar to *E. coli*-LLO/OVA (Figure 56).



**Figure 56. Tumour protection by peptide vaccination with *E. coli*-LLO adjuvant.** Feale C57BL/6 mice received 2 s.c. injections of fixed bacteria ( $10^8$ /mouse with/without 100  $\mu\text{g}$ /mouse peptide) at one-week intervals and were then challenged with B16-OVA ( $5 \times 10^5$  cells/mouse) one week after the last vaccination (5 mice in each group). Tumour volume was assessed by the maximal 2-dimension measurement and expressed in  $\text{mm}^2$ . Results are expressed as mean  $\pm$  SD. \*\*  $p < 0.01$

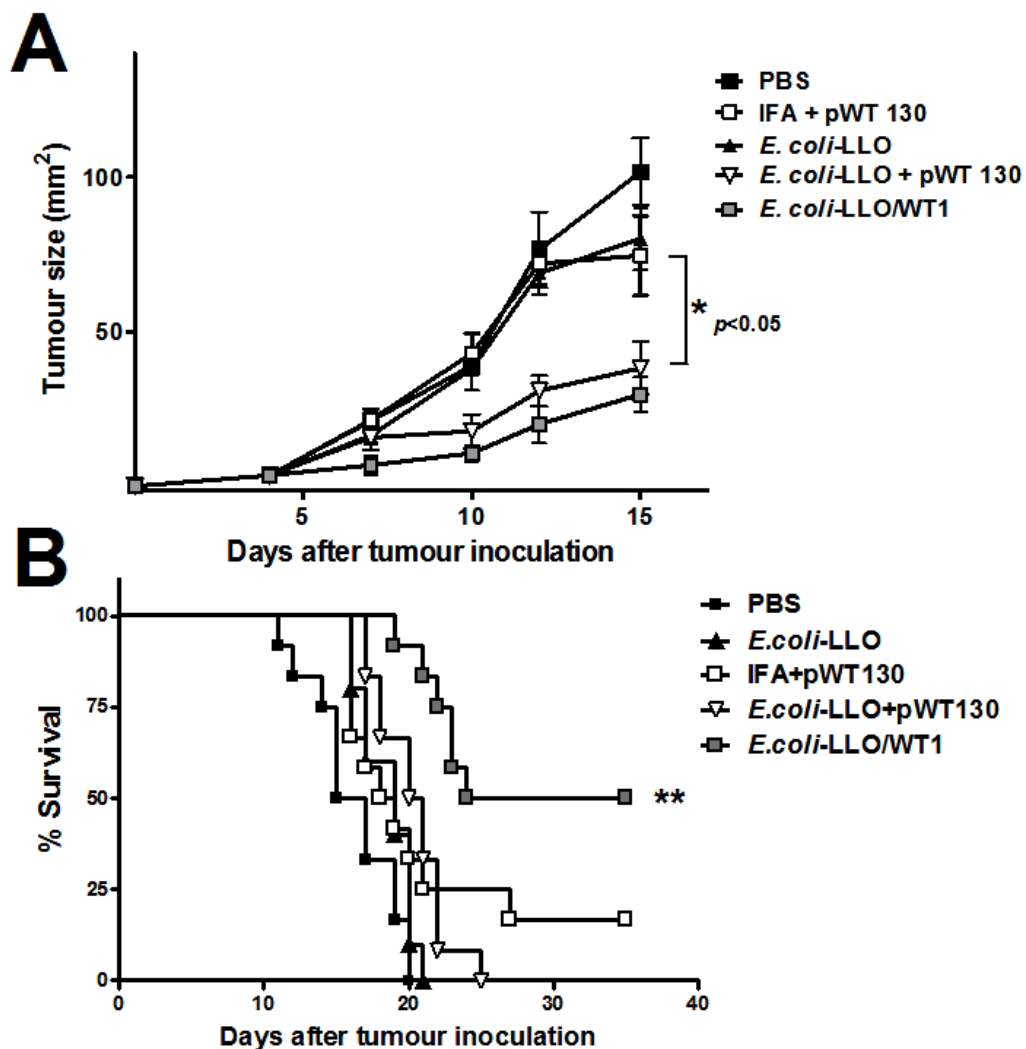
In the model of clinically relevant tumour antigen-WT1, most of the mice were protected from lethal tumour challenge in the group of *E. coli*-LLO/WT1 or *E. coli*-LLO with *pWT*130 (Figure 57). By contrast, in PBS- or *pWT*130 with IFA-vaccinated animals, no tumour rejection was seen. In the mice vaccinated with *E. coli*-LLO, two out of the six mice showed tumour regression but the tumours continued to grow after 15 days in others.



**Figure 57. Preventive vaccination with *pWT*130 with *E. coli*-LLO adjuvant in tumour inhibition.** In the preventive model, female C57BL/6 mice received 3 subcutaneous injections of PBS, *pWT*130+IFA, *E. coli*-LLO, *pWT*130+*E. coli*-LLO, or *E. coli*-LLO/WT1 ( $10^8$  bacteria/mouse, 100  $\mu$ g peptide/mouse) at one-week intervals and were then challenged with MBL2-WT1 ( $5 \times 10^6$  cells/mouse) one week after the last vaccination (6 mice in each group). The effect was represented by tumour volume assessment (A) (mean  $\pm$  SD, \*  $p < 0.05$ ) and

survival (B) (\*\*  $p < 0.01$ ). This experiment has been repeated once with similar results and the survival (B) represented the pooled data from 2 experiments.

In the therapeutic setting (Figure 58), the mice vaccinated with *E. coli*-LLO/WT1 and *E. coli*-LLO with pWT130 demonstrated superior tumour growth inhibition responses at early time-points but this effect did not provide a significant benefit on survival in mice vaccinated with *E. coli*-LLO with pWT130. In contrast, the *E. coli*-LLO/WT1 vaccine caused tumour regression in 50% of the mice.



**Figure 58. Therapeutic vaccination with pWT130 with *E. coli*-LLO adjuvant in tumour rejection.** In the therapeutic model, female C57BL/6 were first inoculated with MBL2-WT1 ( $5 \times 10^6$  cells/mouse) on Day 0 and vaccinations with PBS, pWT130+IFA, *E. coli*-LLO,

pWT130+*E. coli*-LLO, or *E. coli*-LLO/WT1 ( $10^8$  bacteria/mouse, 100 µg peptide/mouse) were given on Days 5, 10, and 15. The effect was represented by tumour volume assessment (A) (mean  $\pm$  SD, \*  $p < 0.05$ ) and survival (B) (\*\*  $p < 0.01$ ). This experiment has been repeated once with similar results and the survival (B) represented the pooled data from 2 experiments.

## 6.4 CONCLUSION

In this chapter, I have demonstrated that *E. coli*-LLO *per se* can act as an immunostimulatory adjuvant. It can be exploited in conjunction with peptide vaccination with beneficial effects compared to peptide vaccination with/without IFA. In both the model tumour antigen and real tumour antigen settings, applying *E. coli*-LLO with single antigenic (CTL) epitope vaccination can help to potentiate the cytokine responses and tumour rejection.

## CHAPTER 7: DISCUSSION

### 7.1 SPOTLIGHT OF T<sub>REG</sub> INHIBITION IN *E. COLI*-LLO VACCINATION

Naturally-occurring regulatory T (Treg) cells have been shown to suppress immune responses to self-antigens, thereby limiting autoimmunity. In the case of tumours, where immune responses to self-antigens are beneficial and lead to elimination of the tumour, such suppressive activity is detrimental. In the light of recent evidence, it would seem that the most promising and synergistic approaches for cancer immunotherapy will be ones designed to augment specific anti-tumour immunity while simultaneously reducing the effect of immunoregulatory mechanisms *in vivo*. This type of strategy has already been tested in humans (O'Mahony, Morris et al. 2007; Ribas, Hanson et al. 2007). Therefore, the strategies which modulate Treg cells hold great promise for immunotherapy of cancer and the interaction between vaccine and Treg cells will be examined upon the development of newer generations of cancer vaccines.

This project demonstrated that the presence of LLO in a vaccine formulation can inhibit Treg suppressive functions. Using the model tumour antigen OVA, the results show that *E. coli*-LLO/OVA is a powerful tool for successful anti-tumour vaccination, through its ability to generate specific CTL and to affect Treg function. Recombinant *E. coli* have already been described as protein delivery vectors for professional phagocytic cells (Higgins, Shastri et al. 1999; Radford, Higgins et al. 2002), and presentation of the OVA epitope on MHC class I by *E. coli*-LLO/OVA has been shown to be orders-of-magnitude more efficient than *E. coli*-OVA strains (Nitcheu-Tefit, Dai et al. 2007). Therefore, the difference in efficacy of the two strains may be

attributed to a difference in efficiency of MHC class I presentation on antigen-presenting cells. This hypothesis is partly confirmed by the observation that higher frequencies of OVA-specific CD8<sup>+</sup> T cells were found in *E. coli*-LLO/OVA vaccinated animals. However, additional data demonstrated that the removal of CD4<sup>+</sup> or CD25<sup>+</sup> T cells at the priming stage allowed the activation of OVA-specific CD8<sup>+</sup> T cells in *E. coli*-OVA-immunised animals, resulting in efficient tumour immunity. Although conventional CD4<sup>+</sup> T cell participates in memory T cell generation during vaccination, the absence of this pathway is more than compensated for by the potency of Treg depletion.

To mediate anti-tumour effects *in vivo*, T cells, especially specific CTLs, of sufficient avidity for recognition of tumour antigen, must present in sufficient quantities, traffic to the tumour site, extravasate from the circulation, and then mediate effector function to eradicate the cancer cells (Gunn, Zubair et al. 2001). The results from the current study showed equivalent anti-tumour effects and IFN- $\gamma$  responses in both groups (*E. coli*-LLO/OVA and *E. coli*-OVA) after removal of a specific group of cells, thus reinforcing the notion that overcoming immune tolerance is required in cancer immunotherapy.

## **7.2 THE IMPACT OF BACTERIAL VACCINE ON TREG**

In a previous report (Gunn, Zubair et al. 2001), a recombinant *Listeria monocytogenes* strain that expresses and secretes the human papilloma virus E7 protein fused to a non-hemolytic form of LLO (*Lm*-LLO-E7) was shown to be effective against established E7-expressing tumours. It is interesting that no protection was observed with a strain that expresses and secretes E7 alone, not fused to LLO, (*Lm*-

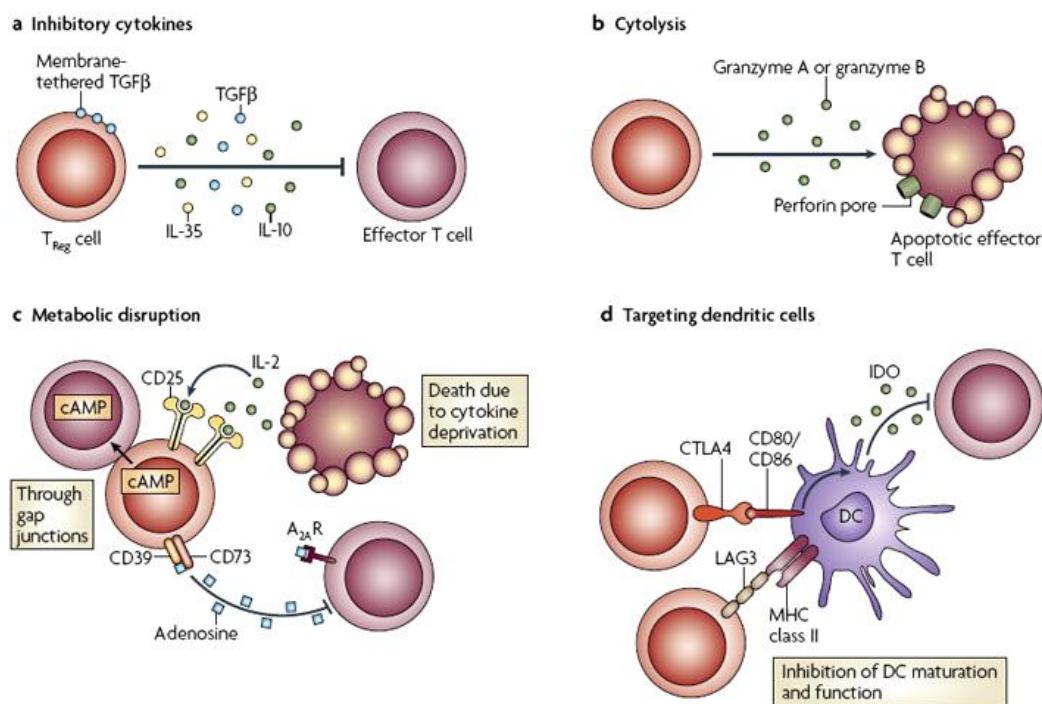


E7) and depletion of CD4<sup>+</sup>, CD25<sup>+</sup> cells, or TGF-β turned *Lm*-E7 into an effective treatment (Hussain and Paterson 2004). Both recombinant *Listeria* vaccines, *Lm*-LLO-E7 and *Lm*-E7, induce measurable anti-E7 CTL responses. The authors found increased numbers of CD4<sup>+</sup>CD25<sup>high</sup> in the spleens and tumours of *Lm*-E7-vaccinated mice compared to *Lm*-LLO-E7-immunised animals and no difference in the Treg suppressive activity. In a more recent study by Shahabi et al., immunisation with *Lm*-LLO-PSA, which encodes the prostate specific antigen (PSA), caused a decline in Treg prevalence in the tumour-infiltrative lymphocytes but not in spleens (Shahabi, Reyes-Reyes et al. 2008). Therefore, *Listeria*-based vaccine can cause a decrease in Treg allocation to tumours in a non-antigen specific manner. To date, there are only limited studies regarding the factors affecting Treg trafficking to tumour. but none has proposed the mechanisms by which bacterial vaccine affects Treg prevalence or trafficking into the tumour microenvironment (Curiel, Coukos et al. 2004; Wei, Kryczek et al. 2007; Haas, Schopp et al. 2008).

### **7.3 MECHANISM INVOLVED IN TREG SUPPRESSION**

Significant progress has been made over the past few years in defining the mechanisms that Treg cells use to mediate their suppressive function. The main mechanisms include (1) secretion of inhibitory cytokines (non-contact), (2) direct cytotoxicity of effectors (contact), (3) local metabolic disruption, and (4) modification DCs co-stimulation (Figure 59) (Vignali, Collison et al. 2008). However, many questions remain unanswered. First, are there more undiscovered mechanisms and/or molecules that mediate Treg suppression? It is becoming clear that the transcriptional landscape of Treg cells is very different from naïve or activated

effector T cells, with literally thousands of genes differentially regulated (McHugh, Whitters et al. 2002). Although it seems unlikely that all or many of these will be important for Treg-cell function, it is quite possible that a few undiscovered genes might be important. It should be noted that although we are discussing functions here, it is clear that some of these molecules may also be essential in Treg-cell homing, homeostasis and other key functions, which might indirectly influence Treg-mediated suppression *in vivo* without directly contributing to their inhibitory activity. Of course, it is also possible that some of these unknown molecules may represent more specific markers for the functionality of Treg *in vivo*, reflecting the status of immune tolerance. In addition, as a particularly important issue for the analysis and use of human Treg cells, we may be able to characterise and separate Treg cells by these unknown molecules.



**Figure 59. Depiction of the various regulatory T (Treg)-cell mechanisms centred around four basic modes of action.** (a) Inhibitory cytokines include IL-10, IL-35 and TGF-β; (b) Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms; (c) Metabolic disruption includes high-affinity CD25 (also known as IL-

2 $\alpha$ )-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A2AR)-mediated immunosuppression; (d) Targeting dendritic cells (DCs) includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3; also known as CD223)–MHC-class-II-mediated suppression of DC maturation, and CTLA4–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs. (Rubtsov, Rasmussen et al. 2008)

At present, it remains difficult to assess which molecular marker is the most important one. Recent studies using mutant mice with a regulatory component specifically deleted in Treg (IL-10) (Rubtsov, Rasmussen et al. 2008) suggest that Treg utilise multiple means to restrict immune responses. It almost goes without saying that, although defining the Treg mode of action is of great academic importance, it is also imperative to develop effective approaches for the manipulation of Treg cells. Given the capacity of Treg to block effective anti-tumour immunity, it seems probable that a clear understanding of how Treg lose their suppression will lead to the development of therapeutic interventions.

#### **7.4 HYPOTHESIS ON HOW LLO INFLUENCES TREG FUNCTION**

The recombinant *Listeria* vaccines (Gram-positive) are likely to reach the cytosol intact and will actively secrete either E7 and LLO-E7 proteins, while in the case of *E. coli*-LLO/OVA (Gram-negative), LLO perforates the lysosomal membrane and allows the release of the bacterial contents into the cytosol (LLO is not fused to the antigen and lacks its secretion signal sequence).

The key question highlighted in my work is how the expression of LLO in the bacterial vaccine affects the functionality of Treg. A hypothetical mechanism of action involves the binding of PAMPs to members of an emerging family of intracellular

receptors, NOD-LRRs (nucleotide-binding oligomerisation domain-leucine-rich repeats), that may sense intracellular pathogens (Inohara, Chamaillard et al. 2005; Martinon and Tschopp 2005). NOD-LRRs are involved in the regulation of apoptosis and inflammation and have been linked to chronic inflammatory disorders (Inohara and Nunez 2003). Thus, activation of this pathway may result in the inhibition of Treg suppression. Another potential mechanism is that the bacterial products could bind to the cytosolic TLRs since there is more and more evidence to support the fact that TLR ligands can directly or indirectly abolish Treg suppression *in vitro* or *in vivo* (Peng, Guo et al. 2005; Wang, Miyahara et al. 2008). In addition, one previously unrecognised action is the cytoplasmic dissemination of perforating LLO. In the *Listeria* infection model, Gekara et al demonstrated the depletion of intracellular  $Ca^{2+}$  stores by LLO and desensitisation of immune cells resulted from impaired  $Ca^{2+}$ -dependent signalling (Gekara, Groebe et al. 2008).

## **7.5 EXPLORING THE SUPPRESSION SIGNATURE OF TREG BY MICROARRAY ANALYSES**

Since the discovery of IL-2 receptor alpha-chains (CD25) as a Treg marker in 1995 (Sakaguchi, Sakaguchi et al. 1995), several other markers related to suppressive function have been reported such as Foxp3, CTLA-4, GITR, PD-1, OX40, CD101, and IL-35 (Eikmans, Roos-van Groningen et al. 2005; Santamaria-Kisiel, Rintala-Dempsey et al. 2006; Lehner 2008). Interestingly, array analyses demonstrated that there is no significant expression difference of the genes previously postulated between these two groups of Treg, implying that other genes may be responsible for Treg functional reversal by bacterial vaccine. Inflammation-related genes, including

S100 A8 (Calgranulin 8), S100 A9 (Calgranulin 9), Lipocalin-2 (LCN-2), Chitinase 3-like-3, Lactotransferrin, Cathelicidin antimicrobial peptide (Camp), and Secretory Leukocyte protease inhibitor (SLPI), were found up-regulated in non-functional Treg (i.e, Treg from *E. coli*-LLO-immunised mice). Of note, S100 A8/A9 were found to be substantially up-regulated genes in Treg from *E. coli*-LLO-immunised animals (greater than 2 log differences) in all repetitive analyses. The heterodimers have been suggested as an indicator of early rejection in renal grafts. Another study by Vogl et al demonstrated that this complex can amplify the endotoxin-triggered inflammatory responses of phagocytes and could be the endogenous ligand of TLR4 (Vogl, Tenbrock et al. 2007). Although limited evidence supports the pro-inflammatory role of S100 A8/A9 complexes, there is no proposed direct correlation between S100 expression and the development of dysfunctional Treg. Other inflammatory genes upregulated in non-functional Treg, such as Lipocalin-2, Chitinase 3-like-3, and Lactotransferrin, have been found up-regulated in certain infectious or autoimmune disease (Legrand, Ellass et al. 2005; Rubinstein, Pitashny et al. 2008) but again no direct effect on Treg has been demonstrated.

When examining other differentially-expressed cytokine/chemokine genes, several genes were identified which could be relevant to the Treg functional alteration, such as IL-1 $\beta$ , IL-10, IL-22, IL-17, TGF- $\beta$ i, CCR1 and CXCL2. Even though different levels of IL-10 and TGF- $\beta$  secretion in activated Treg were observed *in vitro* (Figures 19 and 20), the levels of these cytokines was not modified *in vivo* (Table 5). IL-17 has recently been widely described for its capability of bridging the adaptive and innate immune systems. A specific subset of committed helper T cells secreting IL-17 is known as Th17 cells (Ouyang, Kolls et al. 2008). Th17 cells play indispensable roles in graft rejection and autoimmune disease (Afzali, Lombardi et al. 2007) and more

attention has been drawn toward the interplays between Th17 and Treg (Oukka 2007). More recently, *in-vitro* and *in-vivo* experiments have shown that in the stimulation of IL-6 and TGF- $\beta$ , Treg can be differentiated into Th17 or even other T effectors (Radhakrishnan, Cabrera et al. 2008). This points to an interesting issue relevant to our microarray findings about the functional status of Treg and IL-17 gene expression. Meanwhile, skewed Th17 differentiation seems to reduce Treg prevalence within the tumour microenvironment, reflecting the balance of Th17/Treg in the tumour infiltrated lymphocytes (Sfanos, Bruno et al. 2008). Chen et al demonstrated that pertussis toxin can dampen Treg suppression by the generation of IL-6-dependent Th17 cells (Chen, Howard et al. 2007). Recently, evidence suggested that the inhibition of Th17 polarisation may enhance Foxp3 expression in Treg (Elias, Laurence et al. 2008) and suggesting a role in the balance between Treg and Th17 cells.

IL-1 $\beta$ , one of the pro-inflammatory cytokines, has been described for its capability of attenuating CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg function, and escape of CD4<sup>+</sup>CD25<sup>-</sup> autoreactive effectors from suppression (O'Sullivan, Thomas et al. 2006). In conjunction with IL-6 and TNF- $\alpha$ , IL-1 $\beta$  also involve in the Th17 skewed differentiation and reduce the ability of Foxp3<sup>+</sup> Treg to maintain tolerance to self (Kimura, Naka et al. 2007). Within the microarray analysis, many IFN or TNF ligands/receptors have shown different degrees of up- or down regulation in Treg from *E. coli*-LLO-immunised mice. In addition, growth factor (amphiregulin) and other membranous/cytosolic protein (Granzyme B, Neutrophilic granular protein, lysozyme, B-lymphoid kinase, and Cathepsin H) have also come out in the genome-wide screening and the literature has demonstrated their role in anti-microbial responses and immune modulation (Zaiss, Yang et al. 2006; Malmsten, Davoudi et al. 2007). It

is speculated that all the sophisticated cytokine/chemokine networks need to work simultaneously or sequentially rather than single one of them can shut the Treg suppression down. In the case of the non-tolerised immune state, such as cancer eradication following vaccination, graft rejection, or autoimmunity, the key factor affecting Treg may be different. All of these speculations need to be tested in well-controlled *in-vitro* and *in-vivo* settings.

## **7.6 THE ROLE OF LYMPHODEPLETION FOR EFFECTIVE IMMUNOTHERAPY**

Although Foxp3 has been proposed to be the master regulator of Treg cells, controlling the expression of multiple genes that mediate their regulatory activity, this notion has recently been challenged, raising the possibility that other transcriptional events may operate upstream of and/or concurrently with Foxp3 to mediate Treg development (Hill, Feuerer et al. 2007). In mice, the elimination of CD4<sup>+</sup> suppressor T cells, using various strategies, has been extensively reported to enhance anti-tumour immunity (North 1984; North and Awwad 1987; Awwad and North 1988; North and Awwad 1990; Sakaguchi, Sakaguchi et al. 2001; Suttmuller, van Duivenvoorde et al. 2001). Many studies have reported elevated levels of CD4<sup>+</sup>CD25<sup>+</sup> T cells in patients with different types of cancers (Woo, Chu et al. 2001; Liyanage, Moore et al. 2002; Javia and Rosenberg 2003; Curiel, Coukos et al. 2004; Viguier, Lemaitre et al. 2004). Similarly, greater disease burden and poorer overall survival correlated to increased numbers of Treg cells (Ichihara, Kono et al. 2003; Sasada, Kimura et al. 2003; Curiel, Coukos et al. 2004; Beyer and Schultze 2006). These observations have led to the development of new therapeutic strategies aiming at the elimination of Treg cells in cancer patients and, so far, a small number of single clinical trials have been reported, involving an IL-2/diphtheria toxin conjugate (Ontak, Seragen) to target

CD25 at the surface of Tregs (Chong and Morse 2005; Dannull, Su et al. 2005; Kuzel, Li et al. 2007; Mahnke, Schonfeld et al. 2007; Rasku, Clem et al. 2008). When administered to patients with melanoma, this protein depletes the blood of Treg. In most patients (90%), this treatment has resulted in the production of melanoma-specific CD8 T cells.

Another complementary strategy to potentiate the effect of immunotherapy and induce tumour regression is blocking the immunosuppressive CTLA-4 (O'Mahony, Morris et al. 2007; Hodi, Butler et al. 2008). Increasing early clinical trials have demonstrated the feasibility of targeting tumour-infiltrated Treg and enhancing cytotoxic effectors. These studies highlight the potential of manipulating these cells in cancer immunotherapy (Betts, Clarke et al. 2006).

To sum up the notions previously described, manipulating the regulatory arm of the immune system at Treg either quantitatively or qualitatively is feasible to potentiate anti-cancer therapies. However, variable degrees of auto-immunity, albeit nothing life-threatening has been reported, could occur when immune responses lose their delicate balance. In addition, in humans, the lack of a specific selective marker of Treg is an obstacle to the development of this type of therapeutic approach. Recently, a conditioned-elimination of Treg or temporary reversal of Treg suppression was postulated and it might be of greater safety when applied clinically (Guillot-Delost, Cherai et al. 2008). Moreover, since several categories of Treg have been discovered with different localisations, Treg-targeted intervention should be limited to one specific group, especially the antigen-specific Treg, to maintain tolerance to "real" self-antigen.



## 7.7 CHALLENGES OF WT1-RELATED CANCER VACCINES

WT1 expression in adults appears to be limited to kidney podocytes and some haematopoietic precursors; therefore, oncogenic WT1 expression is a relatively tumour-specific target for therapeutic intervention. A clinical study in leukaemia and MDS patients showed that Th1-biased humoral immune responses against WT1 protein were generated, rather than Th-2 (Wu, Oka et al. 2005). Gaiger and colleagues detected WT1-specific antibodies directed against the WT1 protein in the sera of leukaemia patients (25% with AML and 19% with CML) (Gaiger, Carter et al. 2001). WT1-specific CTL can be expanded from the tumour-draining lymph nodes of breast cancer patients and they can display peptide-specific effector functions (Gillmore, Xue et al. 2006). Oka et al's clinical study showed that the frequencies of WT1-specific CTLs were significantly higher in patients with MDS, AML, breast or lung cancer after WT1 peptide vaccination (Oka, Tsuboi et al. 2004; Gillmore, Xue et al. 2006). These findings indicated that WT1 protein was immunogenic and, thus, could induce WT1-specific T-cells.

The potential MHC-restricted CTL or HTL antigenic epitopes of 9-mer WT1 peptide ( $pWT_{235-243}$ ) had been reported previously and some peptides were applied for cancer immunotherapy (Gao, Bellantuono et al. 2000). Varied clinical responses were obtained in these pilot or phase I clinical trials, furthermore (Mailander, Scheibenbogen et al. 2004; Oka, Tsuboi et al. 2004), one research group in Japan has moved forward to a Phase II clinical trial on account of minute and acceptable toxicity of WT1 peptide vaccination (Morita, Oka et al. 2006). *In-vivo* studies also revealed that mice immunised with WT1 peptide rejected challenges by WT1-expressing tumour cells and survived for a long time with no signs of auto-aggression

by CTL (Oka, Udaka et al. 2000). *In-vitro* expanded WT1-specific CTLs can selectively kill immature CD34<sup>+</sup> cells from CML patients and spare the normal CD34<sup>+</sup> cells (Bellantuono, Gao et al. 2002). A clinical trial of WT1 peptide vaccination using a combination of peptides derived from epitopes recognised by CD4<sup>+</sup> HTLs as well as CD8<sup>+</sup> CTLs (Guo, Niiya et al. 2005) is underway.

Apart from vaccination with WT1 peptide containing immunogenic epitopes, other strategies to augment the immune response to WT1-expressing malignancies have been reported. Nakajima et al reported that the use of WT1 peptide (*p*WT<sub>126-134</sub>) vaccination combined with BCG-CWS leads to rejection of WT1-expressing tumour cells in mice (Nakajima, Kawasaki et al. 2004). Tsuboi and colleagues showed that vaccination with WT1 plasmid DNA can elicit CTL responses specific for the WT1 protein, resulting in the acquisition of rejection activity against challenges of WT1-expressing tumour cells (Tsuboi, Oka et al. 2000). Savage et al reported the potential of immunotherapy with WT1-specific CTLs generated from recombinant HLA-A2 monomers containing single WT1 peptide epitopes as immunogens in allogeneic donors (Savage, Gao et al. 2004).

## **7.8 APPLICATION OF WT1 IN BACTERIAL VACCINES**

WT1 has long been considered as a valid target for cancer immunotherapy and the results of pre-clinical studies using this antigen are summarised in Table 9. In Gaiger et al's study, WT1 peptide vaccination with IFA elicited the production of WT1-specific CTLs and these *in-vitro* expanded CTLs demonstrated specific cytotoxicity to WT1 peptide-pulsed target cells or WT1-expressing tumour cells (Gaiger, Reese et al. 2000). However, the *in-vivo*, protective effect against WT1-overexpressing TRAMP-C following WT1 peptide immunisation was disappointing. The immune responses to

WT1 peptide immunisation might be too weak to elicit significant *in-vivo* tumour immunity. Alternatively, these CTLs may show low avidity to tumour cells. This could be also attributed to low MHC class I expression of TRAMP-C cells, which could pose a problem in clinical studies when MHC class-I are absent or downregulated in certain cancer cells. Another explanation is that those antigen-specific CTLs were somehow anergic or tolerised in the tumour microenvironment. However, in another study by Tsuboi et al (Tsuboi, Oka et al. 2000), vaccination by WT1 plasmid DNA generated CTL activity and significantly prolonged the survival of mice that were challenged by WT1-expressing tumour cells. In human studies using WT1 peptide injection (Oka, Tsuboi et al. 2004), 12 out of 20 cancer patients receiving WT1 peptide injection showed clinical responses with tumour regression and immunologic responses by increasing WT1-specific CTLs (Table 10). Different vaccination strategies might explain this difference in vaccine efficacy even though the same immunotherapy target, WT1, was involved.

Our data showed that vaccination of *E. coli*-LLO/WT1 can elicit significant humoral and cellular immune responses by the generation of specific antibody and inhibition of WT1-expressing tumour challenge. Previous reports have shown that the level of WT1 antibodies correlates with WT1 expression in tumour or disease progression, i.e. the number of leukaemic blasts. Nevertheless, this antibody response did not contribute to any protective effect in rejecting WT1-overexpressing tumour (Wu, Oka et al. 2005). Despite its paucity of clinical relevance, the production of antibody probably correlates with helper T-cell (HTL) stimulation, especially in the *E. coli*-LLO/WT1 vaccination group. Antibody might not have adequate access to the target protein to mediate therapy, however, in order to carry out an effective cancer immunotherapy, MHC class II-restricted responses that elicit anti-tumour CD4+ HTL

will be needed to help the specific CTL by cross-priming. Fujiki et al recently demonstrated that a WT1 helper epitope, WT1<sub>332-347</sub>, could help to improve the efficacy of CTL epitope (WT1<sub>235-243</sub>)-based cancer vaccine targeting WT1 in the clinical setting in terms of specific CTL induction and functional activity (Fujiki, Oka et al. 2007).

Further experiments have verified the tumour-protective effect by means of *in-vitro* antigen-specific T-cell proliferation and WT1-specific CTL killing. Since WT1 is a self protein and is expressed in a limited number of tissues at low level, it is assumed that CTL will be the most effective immune mechanism for vaccine and T-cell therapy. CTL lysis requires the target WT1 peptides to be endogenously processed and presented in association with tumour cell MHC class I molecules. Our study showing CTL lysis of TRAMP-C and MBL2-WT1 provides evidence that WT1 peptides can be presented by MHC class I of tumour cells in high enough amounts to be recognised by WT1-specific CTL, generated by immunisation with *E. coli*-LLO/WT1.

The role of bacteria as an adjuvant in delivering a tumour antigen in cancer immunotherapy has been extensively discussed (Loessner and Weiss 2004; Epaulard, Toussaint et al. 2006; Palffy, Gardlik et al. 2006; Singh and Paterson 2006; Vassaux, Nitcheu et al. 2006). Bacterial vehicles carrying relevant antigens can mount immune responses through APC activation mediated by PAMPs. Apart from this, incorporation of LLO in the vaccine formulation can also lead to improved MHC class I presentation of injected antigen and, therefore, elicit specific CTL response. This may be why the *in-vivo* tumour inhibition was prominent in *E. coli*-LLO/WT1 vaccinated mice but not in *E. coli*-WT1 or WT1 peptides immunised mice. Another study conducted by Ramirez et al has showed that vaccination with WT1 peptide (*p*WT<sub>126-134</sub> or *p*WT<sub>330-337</sub>)-loaded DCs could not elicit WT1-specific CTLs and reject

WT1-tumour growth *in vivo* (Ramirez, Ghani et al. 2007). Collectively, this points to the fact that an appropriate adjuvant and a specific CTL epitope presentation to MHC class-I are of paramount important for effective anti-tumour response.

**Table 10. Summary of published pre-clinical studies involving WT1-related cancer immunotherapy**

Vaccination strategy	CTL- responses	Tumour protection	Humoral responses	
<i>p</i> WT <sub>117-139</sub> in CFA	+	NA	+	(Gaiger, Reese et al. 2000)
LPS-activated spleen cells pulsed with <i>p</i> WT <sub>126-134</sub>	+	+	NA	(Oka, Udaka et al. 2000)
WT1 DNA plasmid	+	+	NA	(Tsuboi, Oka et al. 2000)
<i>p</i> WT <sub>126-134</sub> +BCG-CWS	+	+	NA	(Nakajima, Kawasaki et al. 2004)
WT1 <sub>126-134</sub> or WT1 <sub>330-337</sub> in IFA loaded DCs	-	-	NA	(Ramirez, Ghani et al. 2007)
WT1-TCR gene therapy	+	+	NA	(Xue, Gao et al. 2005)
WT1 <sub>122-140</sub> , WT1 <sub>328-349</sub> , WT1 <sub>423-441</sub> .	+	NA	+	(May, Dao et al. 2007)
WT1 DNA plasmid (encoding epitope WT1 <sub>37-45</sub> )	+	-	+	(Chaise, Buchan et al. 2008)

Abbreviations: CFA: Complete Freund's adjuvant; NA: not available; BCG-CWS:

Mycobacterium bovis bacillus Calmette-Guerin cell wall skeleton;

**Table 11. Summary of published clinical studies involving WT1-related cancer immunotherapy**

Vaccination strategy	Patient No. & type of cancer	Responses	References
Natural, or modified WT1 <sub>235-243</sub> peptide emulsified with Montanide ISA51 adjuvant (Phase I)	20 (breast or lung cancer, MDS, or AML)	12/20 (reduction in leukaemic blasts or tumour sizes/markers)	(Oka, Tsuboi et al. 2004)
WT1 <sub>235-243</sub> peptide emulsified with Montanide ISA51 adjuvant	2 (lung cancers)	1 (tumour marker dropped and transient reduced tumour size)	(Tsuboi, Oka et al. 2004)
WT1 <sub>126-134</sub> with KLH and GM-CSF (Pilot)	1 (relapsed AML)	1 (CR>12 months)	(Mailander, Scheibenbogen et al. 2004)
Modified WT1 <sub>235-243</sub> peptide emulsified in Montanide ISA51 adjuvant (Phase I/II)	10 (solid tumours)	1 PR, 5 SD	(Morita, Oka et al. 2006)
Modified WT1 <sub>235-243</sub> peptide emulsified in adjuvant Montanide	3 (RCC)	2 SD	(Iiyama, Udaka et al. 2007)
WT1 <sub>235-243</sub> peptide emulsified with Montanide ISA51 adjuvant.	1 (relapsed MM)	SD	(Tsuboi, Oka et al. 2007)
WT1 <sub>126-134</sub> and PR1 <sub>169-177</sub> in adjuvant Montanide (Phase I)	8 (AML, CML and MDS in remission)	Safety profiles and CD8 <sup>+</sup> responses	(Rezvani, Yong et al. 2008)

Abbreviations: MDS: myelodysplastic syndrome; AML: acute myeloid leukaemia; RCC: renal cell carcinoma; PR: partial response; SD: stable disease; CR: complete remission; KLH: keyhole limpet hemocyanin; MM: multiple myeloma.

## 7.9 MY PROJECT IN THE FIELD OF WT1 IMMUNOTHERAPY

The identification of TSA recognised by CTL has formed the basis for the design of T cell-based immunotherapeutic cancer vaccines. A growing number of phase I and II clinical trials, which are mainly targeted at melanoma antigens, have been reported over the last decade and are still in development. WT1 is a good TSA for cancer immunotherapy as there is accumulating evidence that WT1 is overexpressed in most leukaemias and various epithelial solid tumours and poorly expressed in normal tissues. Thus, several groups have identified the potent immunogenic peptides of WT1 for HLA-A2 and HLA-A24. They could elicit efficient CTLs against WT1-positive tumour cells *in vitro* (Ohminami, Yasukawa et al. 2000; Azuma, Makita et al. 2002; Bellantuono, Gao et al. 2002; Makita, Hiraki et al. 2002). In particular, the peptide WT1<sub>235-243</sub> with modification of one amino acid has been applied in phase I/II clinical trials with promising results (Table 2) (Oka, Tsuboi et al. 2004; Morita, Oka et al. 2006). However, this immunogenic epitope can only be applied in an HLA-restricted manner (MHC-A\*2402). In Gao's study (Gao, Bellantuono et al. 2000), only the WT1-stimulated allo-HLA-restricted CTL lines can selectively eliminate CD34+ leukaemic cells. The limitations of traditional peptide vaccines are that the antigenic epitope needs to be identified and it may vary among different subjects with different karyotypes. In this regard, our approach that delivers the entire antigenic protein potentially provides a more generally-applicable strategy.

Among the previous publications on WT1 CTL epitopes, pWT126 and pWT235 have been studied for HLA-A\*0201-positive patients. Not only are both peptide sequences identical in humans and mice, but also the vaccination of these peptides can effectively elicit specific CTLs (Oka, Udaka et al. 2000) and caused tumour



regression or disease remission in clinical studies (Mailander, Scheibenbogen et al. 2004; Oka, Tsuboi et al. 2004). In addition, pWT126-specific CTLs could be expanded from tumour-draining lymph nodes in breast cancer patients and acted as effector cells (Gillmore, Xue et al. 2006). In agreement with Gaiger et al's study (Gaiger, Reese et al. 2000), only vaccine containing one 9-mer peptide, pWT130, was able to elicit specific CTL capable of lysing cancer cells expressing WT1, demonstrating that pWT130 is a naturally processed WT1 epitope for B6 mice. In contrast to our data, Gaiger et al reported a lack of anti-tumour activity upon pWT<sub>130-138</sub> vaccination (Gaiger, Reese et al. 2000), highlighting the importance of the bacteria and the LLO as adjuvants.

My project, aiming at the characterisation of the main antigenic motif involved in *E. coli*-LLO/WT1-mediated anti-tumour effect demonstrated the key role of the 9 mers motif NAPYLPSCS (pWT<sub>130-138</sub>). Although my observation pointed to a specific epitope, pWT130, I did not exclude the possibility of other B6 CTL peptides. In another report by Kobayashi et al (Kobayashi, Nagato et al. 2006), pWT<sub>124-138</sub> and pWT<sub>247-261</sub>, were shown to induce peptide-specific HTL, which were restricted by frequently-expressed HLA class II alleles (HLA-DR53). The CTL peptide we identified, pWT<sub>130-138</sub>, is included in one of their proposed WT1 HTL epitopes. Therefore, it might partly explain why *E. coli* LLO/WT1 vaccination in our animal study can elicit effective cellular and humoral immune responses and also, in one CTL assay, we observed a moderate CTL killing in pWT423-stimulated cells (Figure 27). In this context, T cells responding to the helper T-cell epitopes provided further help for eliciting CTL responses and eradicating tumour cells. However, we could not prove its HTL stimulation in a CD4 subset proliferation assay.

## 7.10 POTENTIAL ADJUVANT ROLE OF *E. coli*-LLO

The role of bacteria as a vector or an adjuvant for cancer immunotherapy has been extensively discussed (Loessner and Weiss 2004; Epaulard, Toussaint et al. 2006; Palffy, Gardlik et al. 2006; Singh and Paterson 2006; Vassaux, Nitcheu et al. 2006). In many cases, bacteria express the tumour antigen, target antigen-presenting cells and the adjuvant effect is provided by pathogen-associated molecular patterns (PAMPs). Likewise, the *E. coli*-LLO bacteria were used as a vehicle which expresses the target antigen and is delivered to the host. I have demonstrated that in the absence of an antigen, *E. coli*-LLO immunisation could lead to loss of suppression of Treg (Figures 15 and 20). Having determined the main antigenic motif involved in *E. coli*-LLO/WT1 antitumour activity, the next step was to assess whether this activity could be mimicked by injection of the fixed bacteria expressing LLO and the peptide *p*WT<sub>130-138</sub>. Vaccination, followed by challenge with WT1-expressing cells, demonstrated that injections of the peptide and the fixed *E. coli*-LLO were sufficient to obtain a full-protection against MBL2-WT1 cells (Figure 58). In treatment protocols, injection of *E. coli*-LLO and *p*WT<sub>130-138</sub> resulted in a reduction in tumour growth rate comparable to that obtained with *E. coli*-LLO/WT1 but no overall statistically-significant effect on survival of the animals (Figure 59), while treatment with *E. coli*-LLO/WT1 resulted in a 50% survival of the animals. Considering the superior adjuvant effect obtained upon *E. coli*-LLO injection and the lack of apparent toxicity observed in the mouse model presented here, we advocate the utilisation of either fixed *E. coli*-LLO in conjunction with WT1 antigenic peptides relevant to the HLA haplotype of the patients targeted or fixed *E. coli*-LLO/WT1 in immunotherapy against haematological malignancies. Moreover, since a single peptide with bacterial vaccine

did have a significant effect in tumour protection but not as good as *E. coli*-LLO expressing full antigen, a mixed series of relevant HLA-restricted HTL and CTL epitopes in conjunction with *E. coli*-LLO vaccination might produce a better anti-tumour response. The results obtained in this project will facilitate the translation of this work to human studies by combining antigenic motifs relevant to specific human HLA haplotypes with the adjuvant effects of *E. coli*-LLO.

### **7.11 THE CHALLENGES OF THERAPEUTIC VACCINE**

In many previous pre-clinical studies of cancer vaccines, anti-tumour vaccines are effective in preventing a subsequent tumour challenge in animals using many different fast-growing and aggressive mouse tumours. Vaccines being tested in these models consisted of live, irradiated or genetically modified tumour cells, dendritic cells, proteins, peptides or naked DNA. Each of these vaccine preparations can be given alone or combined with cytokines and co-stimulatory factors. In mice, effective immunity is often elicited and a successful pre-immunisation against almost any kind of tumour seems to be feasible. Previous experiments involving model antigen in *E. coli*-LLO/OVA vaccine have shown a significant anti-tumour effect following B16-OVA challenge in either therapeutic or preventive setting. In real antigen models, WT1-expressing *E. coli*-LLO also can cure 50% of tumour-harboured animals and lead to increased long-term survival. However, in the experimental model, therapeutic vaccines were administered shortly after tumour implantation, that is, the animals were not an authentic model of immuno-compromised state. The theory of immunosurveillance suggests three phases in the evolution of a tumour, in relation to the immune system: elimination, whereby the immune system is capable of detecting

and eliminating the developing tumour; equilibrium, during which tumour cells maintain a dynamic equilibrium with the immune system and form new variants; and escape, when variant tumour cells successfully evade the immune response and grow into a clinically detectable tumour. This theory suggests that most tumours have evolved the ability to evade the immune system by the time they become clinically detectable (Dunn, Old et al. 2004). Implantable murine tumours may not be an adequate model for human cancer because the oncogenic events that drive the cancers and the relation between the cancer cells and the tumour milieu may not be the same as in spontaneously-arising tumours. The use of human tumour xenografts requires implantation into severe immunodeficient mice, obviously devoid of an immune system. Regeneration of a human immune system in these mice is technically challenging, and current approaches may not provide a fully functional immune response to test immune sensitisation approaches. In this context, one of the limitations of the implanted tumour model is that, a lethal dose of tumour cells is injected. The pathophysiologic situation is different, with a few cells evading immunosurveillance and creating a tumour.

In cancer patients, we can expect that the efficiency of induction of antigen-specific T-cells will be reduced, mainly due to a global reduction of the T-cell repertoire as a consequence of tumour growth, the expansion of regulatory cells, and/or following cytotoxic chemotherapy. They may diminish the frequency or reactivity of tumour-specific T cells. Results from testing the anti-tumour effect in existing tumour models are critical for the rationale in moving forward to clinical trials in cancer patients, but the effect might be suboptimal. Other combined strategies, such as *ex-vivo* expansion or adoptive T cell therapy, may be required when applied to cancer patients. Since experimental data suggest that vaccination is more likely to be

effective on small tumour burden, such as a minimal residual disease after conventional treatments, or tumours at an early stage of disease, better selection of patients will allow more reliable clinical results to be obtained (Bocchia, Bronte et al. 2000).

Another critical area of focus is the immunologic monitoring of patients. To date, there has been a lack of consensus on minimally-required assays, and standard operating procedures for assays in clinical trials (Keilholz, Weber et al. 2002); this has limited our ability to compare the results of trials, and to discover the basis of clinical benefit in the small fraction of patients who appear to be successfully vaccinated. Despite past disappointments, novel immunotherapeutic approaches to overcome tumour-induced immune suppression are generating results that warrant renewed enthusiasm and have the potential to significantly alter the outcome of advanced cancer.

## **7.12 DURABILITY OF MEMORY CTLs**

Most experiments described here were conducted in tumour challenge close to vaccinations. Another important point to be explored is the effect of a gap between vaccination and challenge. Induction of efficient long-term immune memory is the aim of all vaccination protocols. The factors required to maintain memory cell populations have been controversial. In mice, memory T cell survival does not require the persistence of cognate Ag and this notion may be supported in patients infected with HIV (Ogg, Jin et al. 1998). In contrast, persistent infection of mice with lymphocytic choriomeningitis virus (LCMV) resulted in selective deletion or anergy of high avidity memory CTL. Administration of LCMV vaccines successfully induced lytic MHC-

restricted CTL in the persistently virus-infected mice; however, these CTL were of low avidity and could not clear the viral infection (Hou, Hyland et al. 1994).

The effector CD8<sup>+</sup> T cells induced by CTL epitope peptides do not last more than about 3 weeks after induction and no functional memory CD8<sup>+</sup> T cells are generated (Janssen, Lemmens et al. 2003; Kaech and Ahmed 2003). It is held that simultaneous induction of CD4<sup>+</sup> T cells by incorporation of peptides containing T-helper epitopes in the vaccine at the time of primary vaccination is necessary for the induction of long-lived functional memory CD8<sup>+</sup> T cells (HTL-dependent) (Agnellini, Wiesel et al. 2008). The effect of inducing durable memory-CTL by *E. coli*-LLO/WT1 vaccination or peptide with *E. coli*-LLO also needs to be documented before clinical application in cancer patients.

### **7.13 AUTO-IMMUNE REACTION RELATED TO BREAKING SELF-TOLERANCE**

Immunotherapy aims to harness the immune system to impact the treatment of a wide variety of diseases. However, for any specific therapy to be successful, it is critical that it directs a specific immune attack to the disease while protecting the host from aggressive autoimmunity. This selectivity is particularly challenging in the case of cancer, where tumour growth is driven by mutations or abnormal expression of normal cellular proteins. To the host immune system, these “tumour-associated antigens” are likely to be recognised as an extension of self, allowing tumour protection via active immune tolerance. On the contrary, serologic and clinical manifestations of autoimmunity might occur if the immunotherapy is effective against those tumour antigens. For example, the appearance of autoantibodies and vitiligo is usually associated with improved survival in melanoma patients following interferon,

peptide vaccine, or Treg depletion treatment (Ribas, Camacho et al. 2005; Gogas, Ioannovich et al. 2006; Slingluff, Petroni et al. 2008).

The WT1 gene is physiologically expressed in some organs such as kidney, spleen and bone marrow. Previous experimental evidence demonstrated that WT1-specific CTLs kill WT1-expressing leukaemic cells, but spare normal haematopoietic cells (Gao, Bellantuono et al. 2000). In mice immunised with MHC class I-restricted WT1 peptides or WT1 cDNA, the WT1-specific CTLs induced killing of WT1-expressing tumour cells, but no evidence of damage to normal tissue was observed (Tsuboi, Oka et al. 2000; Morita, Oka et al. 2006).

There are at least four possible mechanisms by which WT1-specific CTLs can ignore physiologically WT1-expressing normal cells. First, WT1 expression levels in normal cells are lower than those in tumour cells. However, this possibility is unlikely because WT1 expression levels in CD34+ normal haematopoietic progenitor cells are similar to those in leukaemic cells at the single-cell level. Second, expression of MHC class I molecules may be lower in normal cells than in tumour cells. Third, in WT1-expressing normal cells, WT1 peptides may not be presented on MHC class I molecules, or the presentation of WT1 peptides onto the molecules may be weak. The poor presentation of WT1 peptides could be ascribed to differences between normal and transformed cells in the processing of WT1 proteins in proteosomes or in the transport of the processed WT1 peptides onto the cell surface. Fourth, compared to WT1-expressing tumour cells, WT1-expressing normal cells do not, or weakly, express cell surface costimulatory molecules needed for recognition and/or killing by WT1-specific CTLs.

To sum up, even though we did not observe clinically overt auto-immunity in kidney tissues (Dr. J Nitcheu-Tefit, personal communication) in *E. coli*-LLO/WT1-

immunised mice, it is impossible to exclude long-term toxicity of the approach. This issue should be addressed when moving into clinical application.

## **7.14 FUTURE WORK**

### **7.14.1 Clinical Application of *E. coli*-LLO/WT1**

In this thesis, I have clearly demonstrated that pWT<sub>130-138</sub> was consistently the immuno-dominant epitope to stimulate lymphocyte proliferation *in vitro* and induce vigorous specific CTL responses. However, combination of pWT130-138 with other HTL or CTL epitopes may increase this efficacy. Another application of the *E. coli*-LLO adjuvant properties may be their combination with tumour lysates.

My subspecialty is clinical haematology and oncology and most of my patients diagnosed as leukaemia and lymphoma. A certain number of these patients with WT1 over-expression in their tumours will be candidates for WT1 immunotherapy. Even though cytotoxic chemotherapy and stem cell transplantation can cure a large population of patients, some remain resistant to conventional treatments. Therefore, I would be keen to extend my preclinical work to the clinic. When the bacteria express a nearly full-length antigen, the concern of HLA-restriction can be eliminated. An important step is to produce GMP-grade, fixed-bacteria for human application and to start a phase I clinical trial to determine the toxicity and maximal tolerated dose in humans. It is assumed that the toxicity will be minimal by subcutaneous immunisation according to previous human studies involving intravenous injection of Salmonella vaccine (Toso, Gill et al. 2002). In addition to the clinical toxicity profiles, immunologic and tumour responses will be observed in the clinical trial, of particular importance



will be the effect of *E. coli*-LLO vaccination on Treg function. The feasibility of combination strategies with bacterial vaccines may be tested in the long run.

#### **7.14.2 Optimisation of immunotherapy**

Cancer vaccines are often successful at generating elevated numbers of tumour-specific CTLs in peripheral blood; however, these CTLs are usually unable to eliminate tumours even after effective vaccination. Prolonged antigen stimulation might induce deletion of memory CTLs and tolerance compared to short antigen exposure (den Boer, Diehl et al. 2001). Utilisation of adjuvants may serve to stimulate a local immune response leading to recruitment of APCs, but the delivered antigen might be spread out of depot systemically, thus reducing the efficacy of vaccination. In this respect, it is important to find a balance between effective immune response and high yield with long-lasting CTL memory.

Recent evidences suggested that a prime-boost protocol involving sequential administration of different vaccination strategies was therapeutically effective in rejecting tumour cells *in vivo* (Sedegah, Weiss et al. 2000; Meng, Butterfield et al. 2001; Wang, Wang et al. 2005). In the study by Wang et al, priming with plasmid DNA and boosting with antigen-expressing adenovirus induced higher levels of cellular and humoral immunity than either plasmid or adenovirus vaccination alone (Wang, Wang et al. 2005). Since the bacterial vaccine in our study can elicit vigorous CTL responses, we may try to prime first with bacterial vaccine and boost by other strategies, such as plasmid DNA, peptides, or tumour lysate with/without *E. coli*-LLO adjuvant, to maintain the viability of specific CTLs. However, a sustained memory CTLs with effective tumour killing should outweigh the drawback of immune tolerance

from prolonged antigen stimulation. In addition, the local suppressive factors within the tumour microenvironment should be considered even if substantial maintenance of effector memory cells can be achieved.

### **7.14.3 Listeriolysin-O-mediated functional change of Treg**

Evidence from our previous experiments suggested that LLO did impact on the suppressive function of Treg. The data from mRNA expression profiling of Treg cells, regarding the differentially-regulated genes encoding proteins mediating the functional change of Treg cells, provide valuable information. These data have been validated by RT-PCR, and I was able to identify several genes of chemokine/cytokine, growth factors and receptors associated with non-functional Treg. The next step will be to manipulate those genes *in vitro* to determine the effect on Treg function.

Several additional issues still need to be further clarified: (1) Is LLO the effect on Treg cells dose-dependent, (2) does LLO itself triggers the response or is LLO needed simply to allow for cytosolic localisation of bacterial product, (3) the interaction between bacterial product and expression of LLO within the cytosol, (4) the signalling pathway of LLO in the cytosolic compartment. According to the study by Mandal and Lee (Mandal and Lee 2002; Stier, Mandal et al. 2005), effectively priming cellular immunity with a robust CTL response could be achieved in the LLO-liposome system delivering OVA antigen. To elucidate the aforementioned issues, a precisely-controlled LLO concentration and intra-cytosolic delivery of LLO can be achieved in this system. In addition, the signalling pathway of LLO in cytosol can be further studied in the absence of co-stimulatory effect from digested bacterial product, PAMPs, or co-expressed antigen.

Since the LLO-expressing gene, *hly*, was “borrowed” from *Listeria monocytogenes*, the immune responses stimulated by *E. coli*-LLO/OVA and Lm-LLO/OVA vaccination were similar, but not exactly the same (Grosenbach, Barrientos et al. 2001) (D’Orazio, Troese et al. 2006). Cytosolic LLO is directly responsible for triggering an early IFN- $\gamma$  response in both vaccine strategies although it is not yet clear whether LLO plays a direct role in triggering a signal cascade that leads to cytokine production or whether it is required simply to release other bacterial product(s) into the host cell cytosol. To investigate the detailed immune mechanisms of the LLO effect, the use of *L. monocytogenes* or liposome-encapsulated LLO might be considered.

## CONCLUDING REMARKS

Efforts to harness the immune system for cancer therapy have tremendous potential, but immunotherapy faces daunting challenges. To be successful, immunotherapy must overcome a variety of obstacles, including tumour-induced immune suppression. As more and more antigens are identified for various kinds of cancer, bacterial vectors will offer a shuttle to introduce the newly identified antigens to the immune system. Furthermore, it should be possible to coordinately express more than one antigen at a time (i.e., a panel of disease-specific antigens) to mount a complete immune response against a particular disease in one particular vaccination. Although the experiences from LLO expressed in bacterial vaccine have greatly improved the antigen delivery and intracellular processing, the detailed mechanisms involved in LLO-mediated modification of the immune response remain to be characterised. Also, the dosage, route of vaccination, and optimal prime/boost strategy in bacterial vaccine therapy still need to be further defined.

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## Listeriolysin O Expressed in a Bacterial Vaccine Suppresses CD4<sup>+</sup>CD25<sup>high</sup> Regulatory T Cell Function In Vivo<sup>1</sup>

Josianne Nitcheu-Tefit,<sup>\*</sup> Ming-Shen Dai,<sup>\*§</sup> Rebecca J. Critchley-Thorne,<sup>2\*</sup> Francisco Ramirez-Jimenez,<sup>†</sup> Man Xu,<sup>\*‡</sup> Sophie Conchon,<sup>¶||</sup> Nicolas Ferry,<sup>¶||</sup> Hans J. Stauss,<sup>†</sup> and Georges Vassaux<sup>3\*¶||</sup>

CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells (Treg) protect the host from autoimmune diseases but are also obstacles against cancer therapies. An ideal cancer vaccine would stimulate specific cytotoxic responses and reduce/suppress Treg function. In this study, we showed that *Escherichia coli* expressing listeriolysin O and OVA (*E. coli* LLO/OVA) demonstrated remarkable levels of protection against OVA-expressing tumor cells. By contrast, *E. coli* expressing OVA only (*E. coli* OVA) showed poor protection. High-avidity OVA-specific CTL were induced in *E. coli* LLO/OVA-vaccinated mice, and CD8<sup>+</sup> depletion—but not NK cell depletion, abolished the antitumor activity of the *E. coli* LLO/OVA vaccine. Phenotypic analysis of T cells following vaccination with either vaccine revealed preferential generation of CD44<sup>high</sup>CD62L<sup>low</sup> CD8<sup>+</sup> effector memory T cells over CD44<sup>high</sup>CD62L<sup>high</sup> central memory T cells. Unexpectedly, CD4<sup>+</sup> depletion turned *E. coli* OVA into a vaccine as effective as *E. coli* LLO/OVA suggesting that a subset of CD4<sup>+</sup> cells suppressed the CD8<sup>+</sup> T cell-mediated antitumor response. Further depletion experiments demonstrated that these suppressive cells consisted of CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells. We therefore assessed these vaccines for Treg function and found that although CD4<sup>+</sup>CD25<sup>high</sup> expansion and Foxp3 expression within this population was similar in all groups of mice, Treg cells from *E. coli* LLO/OVA-vaccinated animals were unable to suppress conventional T cells proliferation. These findings provide the first evidence that LLO expression affects Treg cell function and may have important implications for enhancing antitumor vaccination strategies in humans. *The Journal of Immunology*, 2007, 179: 1532–1541.

**B**acteria encoding tumor Ags, used as gene or protein delivery systems, have been extensively described in the literature to produce significant and sometimes spectacular antitumor effects in preclinical models (1–13). The general concept is that the bacteria's pathogen-associated molecular patterns act as adjuvants to mount an effective immune response against bacteria-encoded tumor Ags. These pathogen-associated molecular patterns may include among other bacterial components LPS involving TLR4 (14, 15), CpG motifs of bacterial DNA recognized by TLR9 (16), and flagellin, sensed by TLR5 (17).

However, the application of this technology to humans is limited by safety concerns (the bacteria are generally associated with pa-

thologies) and pathogenic genes or proteins present in the bacteria may not be required for the therapeutic effect. To address these concerns, our approach has involved the use of a nonpathogenic strain of *Escherichia coli* that has been engineered to express a minimal number of relevant genes "borrowed" from pathogenic bacteria, necessary and sufficient to confer antitumor activity. So far, the most efficient strain tested is a recombinant *E. coli* that coexpresses the Ag of interest and listeriolysin O (LLO)<sup>4</sup> from *Listeria* (*E. coli* LLO), a member of the pore-forming cytolysins capable of binding and perforating phagosomal membranes at low pH (18, 19). Upon s.c. injection, it is understood that this bacteria is internalized by APCs, taken into the phagosome/lysosome where lysis of the bacterium occurs. Through the pore-forming action of LLO, the cytoplasmic contents of the bacteria can then escape into the cytosol and thereby be processed by the proteasome. In vitro, this LLO-mediated process has been shown to improve MHC class I presentation of the OVA H2-K<sup>b</sup>-restricted epitope SIINFEKL by mouse macrophages (20), mouse bone-marrow-derived dendritic cells (BMDCs) (6), the HLA-A2-restricted MART1<sub>27–35</sub> epitope (21), and the immunodominant epitope of the influenza matrix protein (22) by human monocytes-derived DCs. In vivo, s.c. injection of *E. coli* LLO expressing the model chicken OVA Ag (*E. coli* LLO/OVA) has been shown to trigger a very strong antitumor response against the highly aggressive B16/OVA melanoma cell line (6) (B16-OVA). The antitumor effect of *E. coli* LLO/OVA was Ag specific and far superior to that of *E. coli* expressing OVA only (*E. coli* OVA). Interestingly, the

<sup>\*</sup>Centre for Molecular Oncology, Institute of Cancer, Queen Mary's School of Medicine and Dentistry, London, United Kingdom; <sup>†</sup>Department of Immunology and Molecular Pathology, Royal Free Hospital, London, United Kingdom; <sup>‡</sup>Department of Pathology, Chongqing University of Medical Sciences, Chongqing, China; <sup>§</sup>Division of Haematology/Oncology, Tri-Service General Hospital, National Defense Medical Centre, Taipei, Taiwan; <sup>¶</sup>Institut National de la Santé et de la Recherche Médicale Centre d'Investigation Clinique-04, Université de Nantes, Nantes Atlantique Universités, Centre Hospitalier de l'Université (CHU) Hotel Dieu, Nantes, France; and <sup>||</sup>Institut des Maladies de l'Appareil Digestif, CHU Hotel Dieu, Nantes, France

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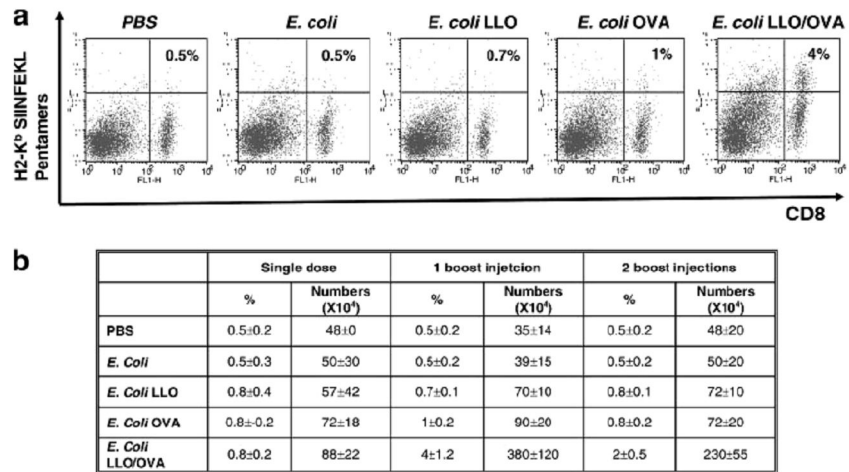
<sup>2</sup> Current address: Division of Hematology, Stanford University School of Medicine, Stanford, CA 94305.

<sup>3</sup> Address correspondence and reprint requests to Dr. Georges Vassaux, Institut National de la Santé et de la Recherche Médicale CIC-04, EE 0502, 3ème étage HNB nord, Centre Hospitalier de l'Université Hotel Dieu, 1 place Alexis Ricordeau, 44035 Nantes Cedex 1, France. E-mail address: georges.vassaux@nantes.inserm.fr

<sup>4</sup> Abbreviations used in this paper: LLO, listeriolysin O; DC, dendritic cell; BMDC, bone marrow-derived DC; LN, lymph node; Treg, regulatory T; T<sub>EM</sub>, effector memory T; T<sub>CM</sub>, central memory T; T<sub>CONV</sub>, conventional T.

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**FIGURE 1.** *E. coli* LLO/OVA vaccination allows the activation of OVA-specific CD8<sup>+</sup>T cells as measured by multimeric MHC-peptide analyses. Mice received several injections of bacteria at a 1-wk interval. Splenocytes were then collected 7 days after each last injection and stained with the SIINFEKL/H-2K<sup>b</sup> pentamers. *a*, Representative experiment depicting percentages of CD8<sup>+</sup>, pentamer-positive cells gated on live lymphocytes are shown. *b*, The percent and absolute numbers of SIINFEKL/H-2K<sup>b</sup>-specific T cells in the spleen (mean ± SD) after a single vaccination, one or two boost injections are shown. \*\*\*, *p* < 0.001.



therapeutic effect does not require live bacteria as it is also observed when the bacteria are fixed with paraformaldehyde before injection (6, 13).

Considering that improved MHC class I presentation of antigenic peptides is unlikely to be the sole mechanism responsible for the striking difference in efficacy between *E. coli* OVA or *E. coli* LLO/OVA vaccines, the aim of the present study was to determine how the expression of LLO in the bacteria can turn a marginally active bacterial vaccine (*E. coli* OVA) into a potent antitumor vaccine (*E. coli* LLO/OVA).

**Materials and Methods**

*Animals*

Six- to 8-wk-old female C57BL/6J mice (20–25 g) were obtained from Harlan Breeders and kept in a germfree environment with irradiated food and acidified water ad libitum. Experiments were conducted after appropriate ethical approval and licensing was obtained in accordance with the U.K. "Guidance on the Operation of Animals (Scientific Procedure) Act 1986" (HMSO, London, U.K.).

*Peptides*

OVA<sub>257–264</sub> SIINFEKL peptide was synthesized at >95% purity. The peptide 130 (NAPYLPSCL) is derived from WT1 protein and was used as an irrelevant peptide. Peptides were synthesized by the Peptide Synthesis Laboratories (Cancer Research U.K.).

*Cell lines*

RMA-S cells, derived from RMA (K<sup>b</sup>D<sup>b</sup>) (23), were cultured in complete RPMI 1640 medium containing 10% FCS. B16 and B16/OVA (the B16 melanoma cell line transfected with the OVA gene; CRUK, London, U.K.), were maintained in DMEM containing 10% FCS supplemented with 400 mg/ml G418.

*E. coli immunization/treatment protocols and tumor challenge*

C57BL/6 mice were vaccinated s.c. with several injections doses of 10<sup>8</sup> of either *E. coli* expressing OVA (*E. coli* OVA) or *E. coli* coexpressing LLO and OVA (*E. coli*/LLO/OVA) at a 1-wk interval. Control groups were immunized with *E. coli*, *E. coli* expressing LLO (*E. coli*/LLO), or PBS (see Refs. 6 and 20 for details of the bacteria). Mice were challenged 1 wk after the last vaccination by injection of 5 × 10<sup>5</sup> B16-OVA cells in the tail vein. Following injection, B16/OVA cells home to the lungs where the cells form multiple nodules. Experiments were terminated and the whole cohort was culled when the first animal became visibly unwell (24–28 days following tumor challenge), then the tumor growth was analyzed by counting nodules in the lungs. For survival experiments, challenged mice in a group were individually monitored until signs of sickness were observed. In treatment experiments, mice were first injected i.v. with B16/OVA tumor cells and treated with s.c. injections of the various bacteria 8 and 15 days later.

*In vitro CTL stimulation and CTL assay*

Stimulating cells were prepared by activating syngeneic spleen cells at 10<sup>6</sup> cells/ml with 25 mg/ml LPS and (Sigma-Aldrich) and 7 mg/ml dextran sulfate (Sigma-Aldrich). The cells were collected 3 days after activation, irradiated (3000 rad), and loaded with the peptides.

RMA-S cells were temperature induced for MHC class I expression at 26°C overnight, and binding of the peptides to H-2K<sup>b</sup> and H-2D<sup>b</sup> was allowed to proceed for 2 h at 37°C with the optimal concentration of the peptide determined in pilot experiments. Effectors cells from vaccinated mice were then mixed with stimulators and cultured for 5–6 days before analyzed in standard <sup>51</sup>Cr-release assays (23). Specific killing was calculated as ((experimental release – spontaneous release)/(maximum release – spontaneous release)) × 100.

*Isolation of BMDCs*

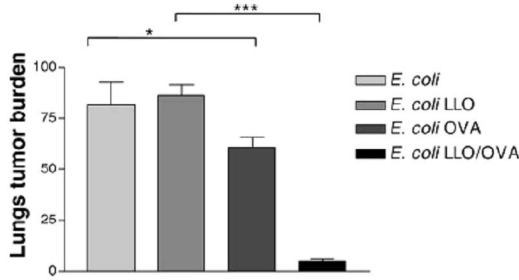
BMDCs were prepared as previously described (6). For loading, 1 × 10<sup>8</sup> of either bacteria was added to 1 × 10<sup>6</sup> BMDCs in a volume of 1 ml in polypropylene tubes in RPMI 1640 medium supplemented with 10% FCS. After 1 h of incubation, Ag-pulsing medium was decanted by several washes before the BMDCs were used for assay.

*Abs and in vivo depletion of T cell subsets*

The anti-CD4 (GK1.5), anti-CD8 (YTS 169.4), and PLTY-1 (isotype control) mAbs were purified from relevant hybridomas (Cancer Research U.K.). Anti-CD25 mAb (clone PC61) and its isotype control (rat IgG1) were purchased from BioExpress. On days 0 and 7, C57BL/6 mice were vaccinated s.c. with 10<sup>8</sup> of either bacterium. Depletion of CD4<sup>+</sup> T cells was achieved at the time of T cell priming by i.p. administration of 300 μg of GK1.5 on days –5 and 10. CD8<sup>+</sup> T cells were depleted by in vivo administration of 400 μg of YTS 169.4-depleting mAbs on days 10 and 17. Control mice were treated with same doses of relevant mAb isotype controls or PBS. Preparatory experiments revealed that YTS 169.4 or GK1.5 mAbs totally depleted CD8<sup>+</sup> or CD4<sup>+</sup> population, respectively, in the spleen, lymph nodes (LN), and peripheral blood 4 days following injection, as measured by flow cytometric analysis, while PLTY-1 mAb control did not deplete either CD4<sup>+</sup> or CD8<sup>+</sup> T cells. To deplete CD25<sup>+</sup> cells, a total of 400 μg of PC61 Ab was injected i.p. on day –1 before vaccinations. Optimal conditions of depletion of these T cell subsets were determined in a preparatory experiment and were shown to totally delete CD25<sup>+</sup> cells in peripheral blood and significantly diminish the percentage of CD25<sup>+</sup> in the spleen and LN for at least 10 days following the injection.

*ELISPOT assay for IFN-γ production*

Ninety-six-well ELISPOT plates (Millipore) were coated with 100 μl/well of 15 μg/ml purified anti-mouse IFN-γ mAb (BD Biosciences) overnight at 4°C. Plates were washed five times with PBS before addition of 8 × 10<sup>5</sup> splenocytes in triplicate wells and 10 μM peptide. Con A was used as a positive control. After 20 h of incubation at 37°C in 5% CO<sub>2</sub>, plates were developed by incubating with 50 μl/well of biotinylated anti-IFN-γ (BD Biosciences) at 1 μg/ml in PBS for 2 h at 37°C. Streptavidin alkaline phosphatase (100 μl; Caltag Laboratories) was added to each well after five



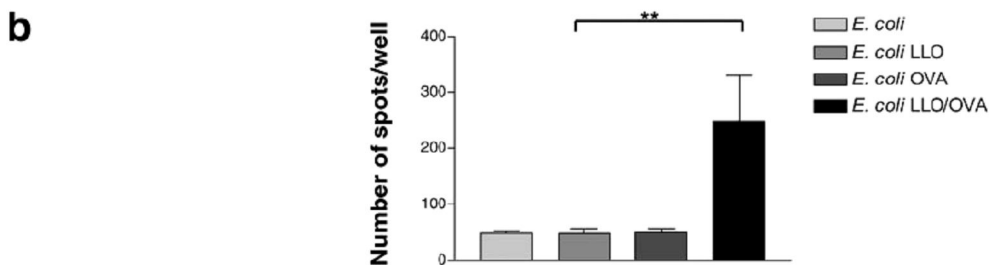
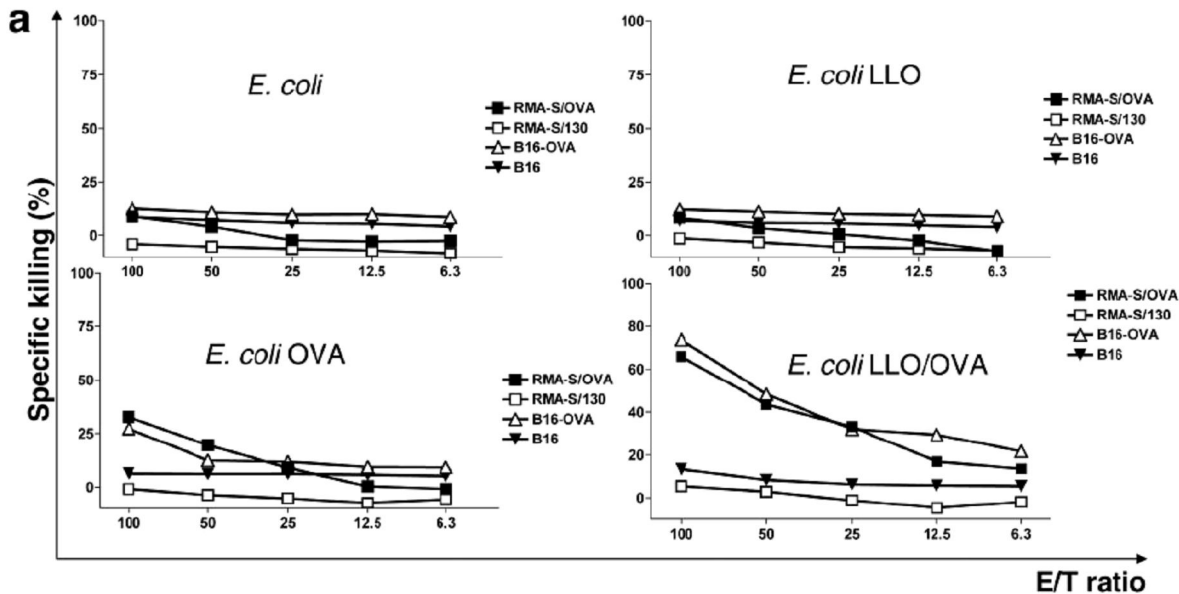
**FIGURE 2.** *E. coli* LLO/OVA vaccine inhibits lungs metastasis induced by i.v. injection of B16 cells expressing OVA. C57BL/6 mice were given two s.c. injections of bacteria at a 1-wk interval, then were challenged a week after the boost injection by tail-vein injection of  $5 \times 10^5$  B16/OVA cells. Animals were sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumor challenge) and the tumor growth was assessed by counting tumor nodules in the lungs. Results are expressed as mean  $\pm$  SD. The experiments presented are representative of at least three experiments involving six animals per groups. \*,  $p = 0.01$ ; \*\*\*,  $p < 0.001$ .

washes and incubated for 1 h at room temperature (RT). The plate was developed using 100  $\mu$ l of alkaline phosphatase-conjugate substrate (BioRad). Spots were counted by an automated ELISPOT reader and a response was considered positive when spot numbers in triplicate assays in the presence of the specific peptide significantly exceeded the cutoff value, corresponding to the number of nonspecific spots in the presence of irrelevant peptide.

#### Abs and pentamers and flow cytometric analysis

Anti-CD3 FITC, Anti-CD4 PerCP, anti-CD8 $\alpha$  PerCP, anti-CD62L allophycocyanin, and anti-CD44 PE were purchased from BD Biosciences. PE-conjugated H-2K<sup>b</sup>/SIINFEKL pentamers were purchased from Proimmune. The Mouse Regulatory T Cell Staining kit was purchased from eBioscience. Single-cell suspension obtained from spleen and LNs were treated in ammonium chloride potassium (ACK) buffer to lyse erythrocytes, washed three times, and resuspended in PBS containing 3% FCS (FACS buffer). The cells were blocked for nonspecific binding with anti-Fc $\gamma$ R2 before being incubated with optimal concentration of appropriate mAbs for 30 min on ice, then washed and resuspended in FACS buffer.

For pentamer staining, the cells were first labeled with the pentamer for 10 min at RT, washed once, then incubated with optimal concentration of anti-CD8. Pentamer staining was analyzed by gating on CD8<sup>+</sup> cells. For regulatory T cell staining with the staining set (eBioscience), cells were treated according to the manufacturer's instructions. Data were collected



**FIGURE 3.** *E. coli* LLO/OVA vaccination generates high avidity CTL responses. *a*, C57BL/6 mice received two s.c. injections of bacteria at a 1-wk interval. Splenocytes prepared from spleens collected 1 wk later were restimulated in vitro with LPS-stimulated spleen cells loaded with the OVA-restricted class I SIINFEKL peptide. Cytotoxicity was measured 6 days later by  $^{51}\text{Cr}$ -release assay against SIINFEKL or irrelevant peptide-loaded RMAS cells as well as B16/OVA and the parental B16 tumor cell lines. Specific  $^{51}\text{Cr}$  release was calculated as described in *Materials and Methods*. Results from individual mice are plotted against the E:T ratio, and are representative of at least two experiments. *b*, Mice received two s.c. injections of bacteria at a 1-week interval. Splenocytes were collected after the boost injection and incubated overnight with the SIINFEKL peptide, and ELISPOT assay was used to measure IFN- $\gamma$  secretion. Results are expressed as mean  $\pm$  SD. Splenocytes from mice that received vaccines coexpressing LLO and OVA induced a significant strong response (\*\*,  $p = 0.0043$ ). The experiments presented are representative of two separate experiments involving three animals per group.

Table I. Mice received two s.c. injections of bacteria at a 1-wk interval<sup>a</sup>

	Spleens			Inguinal LNs		
	CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	CD44 <sup>high</sup> CD62L <sup>low</sup> (%)	CD44 <sup>high</sup> CD62L <sup>high</sup> (%)	CD3 <sup>+</sup> CD4 <sup>+</sup> (%)	CD44 <sup>high</sup> CD62L <sup>low</sup> (%)	CD44 <sup>high</sup> CD62L <sup>high</sup> (%)
PBS	31 ± 3	30 ± 8	59 ± 2	20.8 ± 5	29.9 ± 3	35.5 ± 6
<i>E. coli</i>	27 ± 3	37 ± 3	58 ± 2	31 ± 6	39 ± 25	35 ± 28
<i>E. coli</i> LLO	30 ± 4	29 ± 4	42 ± 11	34 ± 5	30 ± 10	40 ± 8
<i>E. coli</i> OVA	26 ± 3	47 ± 4***	49 ± 4	34 ± 4	46 ± 12	32 ± 13
<i>E. coli</i> LLO/OVA	26 ± 3	54 ± 3***	43 ± 3	27 ± 4	49 ± 20	32 ± 23
	CD3 <sup>+</sup> CD8 <sup>+</sup> (%)	CD44 <sup>high</sup> CD62L <sup>low</sup> (%)	CD44 <sup>high</sup> CD62L <sup>high</sup> (%)	CD3 <sup>+</sup> CD8 <sup>+</sup> (%)	CD44 <sup>high</sup> CD62L <sup>low</sup> (%)	CD44 <sup>high</sup> CD62L <sup>high</sup> (%)
PBS	14 ± 2	14 ± 1	48 ± 3	27.4 ± 3	12.8 ± 2	17.0 ± 1
<i>E. coli</i>	15 ± 2	21 ± 3	53 ± 2	25 ± 5	17.54 ± 10	15 ± 14
<i>E. coli</i> LLO	15 ± 2	8 ± 6	39 ± 8	31 ± 4	10.38 ± 6	22 ± 4
<i>E. coli</i> OVA	16 ± 1	26.73 ± 5***	46 ± 5	25 ± 5	20 ± 7	19 ± 7
<i>E. coli</i> LLO/OVA	17 ± 2	27 ± 3***	48 ± 1	22 ± 4	22 ± 13	18 ± 14

<sup>a</sup> Single-cell suspensions were prepared from the spleens and inguinal LNs collected 7 days after the boost injection then stained with CD3, CD4, CD8, CD62L, and CD44. Percentages of T<sub>EM</sub> (CD44<sup>high</sup>CD62L<sup>high</sup>) and T<sub>EM</sub> (CD44<sup>high</sup>CD62L<sup>low</sup>) cells gated on CD3<sup>+</sup>/CD4<sup>+</sup> or CD3<sup>+</sup>/CD8<sup>+</sup> lymphocytes are shown. Results are expressed as mean ± SD. The experiments presented are representative of two experiments involving six animals per groups. \*\*\*,  $p = 0.0015$  (CD4 T<sub>EM</sub> from *E. coli* OVA- compared to PBS-injected mice); \*\*,  $p = 0.002$  (CD4 T<sub>EM</sub> from *E. coli* LLO/OVA- compared to PBS-injected mice); \*\*\*,  $p = 0.0003$  (CD8 T<sub>EM</sub> from *E. coli* OVA compared to PBS-injected mice); \*\*\*,  $p < 0.0001$  (CD8 T<sub>EM</sub> from *E. coli* LLO/OVA compared to PBS-injected mice).

using a FACSCalibur cytometer (BD Biosciences) and analyzed using CellQuestPro software (BD Biosciences).

#### CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells selection, proliferation, and regulatory cell culture assays

Single-cell suspensions were obtained from the spleens of vaccinated mice. CD4<sup>+</sup> T cells were negatively selected and fractionated into CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> subsets by magnetic Ab cell sorting (MACS; Miltenyi Biotec), using PE-labeled anti-CD25 mAb followed by anti-PE microbeads, according to the manufacturer's instructions. The purity of cells was checked by FACS analysis and >90% of CD4<sup>+</sup> cells were shown to be either CD25<sup>-</sup> or CD25<sup>+</sup>. For proliferation/regulatory assays, 100 × 10<sup>3</sup> responder cells (CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup> T cells) or CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells were cultured in RPMI 1640 medium containing 10% FCS, 50 μM 2-ME with 0.5 μg/ml anti-CD3 mAb (purified anti-CD3; BD Pharmingen) in the presence of 200 10<sup>3</sup> naive irradiated splenocytes. In the MLR, various amounts of regulatory T (Treg) cells were added to 100 × 10<sup>3</sup> responder cells. The cells were cultured for 4 days and proliferation was measured by adding 1 μCi of [<sup>3</sup>H]thymidine (Amersham) to each well for the last 18 h of culture period. The cells were harvested and the thymidine incorporated was determined.

#### Cytokine assays

IFN-γ, IL-2, IL-4, and IL-10 were quantitated in the sera, in culture supernatants of splenocytes, and in *E. coli*-activated BMDCs cultures. Mice received two s.c. injections of the various bacteria, then sera and splenocytes were collected 1 wk after the boost injection. Splenocytes were left unstimulated or restimulated with 10 mM SIINFEKL for 3 days. BMDCs were infected or not with the various *E. coli* and cultured for 48 h. The DuoSet ELISA system (R&D Systems) was used to measure cytokine production according to the manufacturer's protocol.

#### Statistics

Statistical analysis was performed using Prism (GraphPad Software). Dual comparisons were made using the unpaired Student *t* test.

## Results

### Coexpression of LLO in the *E. coli* vaccines allows the expansion of OVA-specific CD8<sup>+</sup> T cells

Animals were given several s.c. injections of 10<sup>8</sup> formaldehyde-fixed *E. coli*, *E. coli* LLO, *E. coli* OVA, or *E. coli* LLO/OVA or PBS, and enumeration of OVA-specific CD8<sup>+</sup> T lymphocytes was performed on splenocytes collected 1 wk after each last injection. Plots depicting percentages of CD8<sup>+</sup>, pentamer-positive cells gated on live lymphocytes are shown in Fig. 1a. Mice receiving *E. coli* OVA vaccines showed little but not significant increase of

SIINFEKL/H2-K<sup>b</sup> specific CD8<sup>+</sup> T lymphocytes above the background levels observed in *E. coli*, *E. coli* LLO, or PBS-vaccinated animals (1 ± 0.2 vs 0.5 ± 0.2% of total splenocytes, respectively). By contrast, *E. coli* LLO/OVA-vaccinated mice induced significant levels of SIINFEKL/H2-K<sup>b</sup>-specific CD8<sup>+</sup> T cells and the highest percentage and absolute numbers (4% of total splenocytes, 3.8 ± 1.2 × 10<sup>6</sup> cells) was achieved following one boost injection (Fig. 1b). As it has been shown that LLO induces apoptosis of infected cells and activated lymphocytes (24, 25), it is likely that multiple boosts may lead to the death of these cells, altering the response. "Prime-boost" vaccines strategies combining naked DNA and *E. coli* may help to amplify Ag-specific immune responses. Thus, vaccines combining LLO and OVA allow the activation of OVA-specific CD8<sup>+</sup> T cells, confirming the previously reported (6) importance of the expression of LLO in the bacterial vaccine.

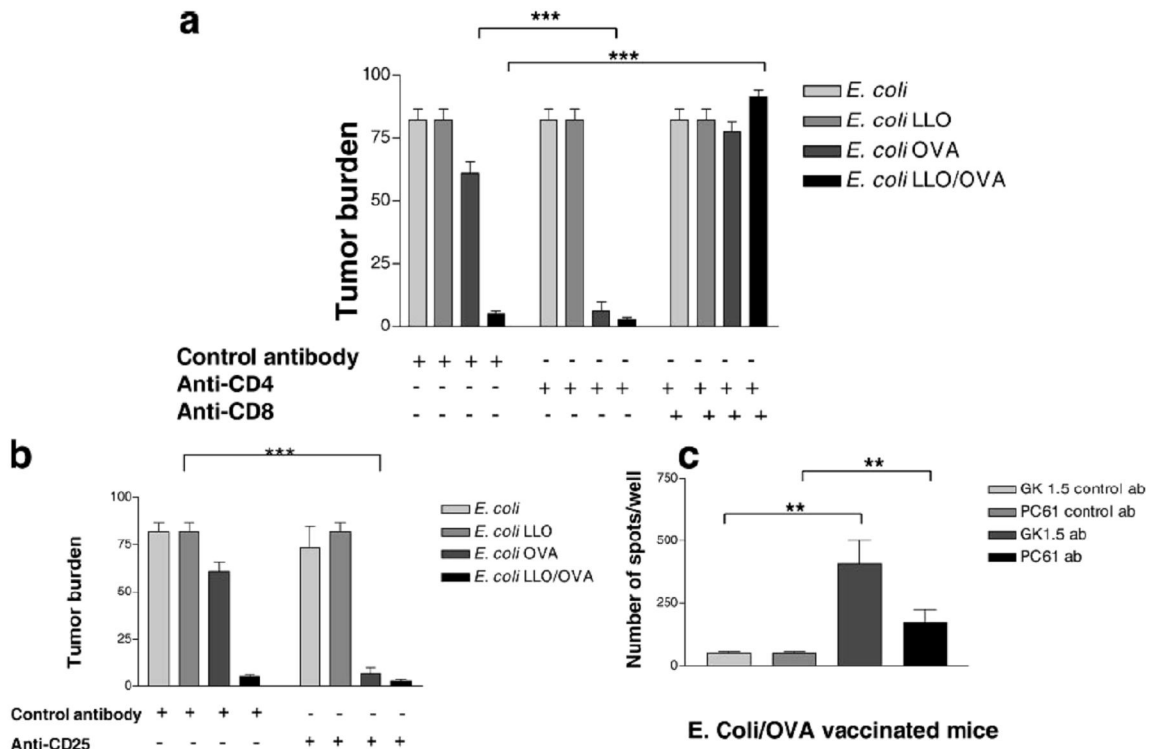
### Vaccination or treatment with *E. coli* LLO/OVA inhibits lungs metastasis induced by i.v. injection of B16 cells expressing OVA

To examine the ability of the vaccines to control tumor, animals received two injections of each of the *E. coli* vaccines at 1-wk interval followed by i.v. challenge of 5 × 10<sup>5</sup> B16 cells expressing OVA (B16/OVA). In this model, B16/OVA cells home to the lungs where the cells form multiple tumor nodules. When the first mice began to show signs of sickness (typically 24–26 days following B16/OVA challenge), the whole cohort was culled and the tumor load was assessed by counting tumor nodules in the lungs.

Fig. 2 shows that the lungs of animals vaccinated with *E. coli* or *E. coli* LLO were heavily colonized by tumors (82 ± 11 and 86 ± 12 nodules, respectively). The burden was modestly but significantly reduced in mice vaccinated with *E. coli* OVA (60 ± 12 per set of lungs,  $p = 0.01$  vs *E. coli*) and dramatically reduced in *E. coli* LLO/OVA-vaccinated animals (5 ± 2,  $p < 0.0001$  vs *E. coli* LLO). The time to lethal tumor burden in *E. coli* OVA-vaccinated mice was extended from 39 to 48 days (compare with *E. coli*-immunized mice) while *E. coli* LLO/OVA vaccine induced complete protection and survival over 200 days in 70% of mice (data not shown).

Importantly, none of these effects were observed upon challenge with parental B16 cells (data not shown), demonstrating that *E. coli* LLO/OVA vaccine resulted in the establishment of an appropriate adaptive OVA-specific immune response.





**FIGURE 4.** CD8<sup>+</sup> T cells are the key mediators for tumor protection and Treg cells prevent their activation in mice receiving the *E. coli* OVA vaccines. *a*, Mice received two s.c. injections of bacteria on days 0 and 7. Depletions were conducted by i.p. injection of GK1.5-depleting mAb (anti CD4) at the stage of T cell priming (on days -5 and 10) in combination or not with i.p. injections of YTS169.4 (anti-CD8) on days 10 and 17. As a control, an irrelevant mAb (PYLT-1) was used. Mice were challenged on day 14 by tail-vein injection of  $5 \times 10^5$  B16/OVA cells and sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumor challenge), then the tumor load was assessed in the lungs. Results are expressed as mean  $\pm$  SD. The experiments presented are representative of two experiments involving six animals per groups; \*\*\*,  $p < 0.0001$ . *b*, Depletion of CD25<sup>+</sup> cells was conducted by i.p. injection of the PC61 mAb on day -1 before s.c. bacterial vaccinations on days 0 and 7. As a control, an irrelevant mAb (RatIgG1) was used. Mice were challenged on day 14 by tail-vein injection of  $5 \times 10^5$  B16/OVA cells and sacrificed when the first mouse showed signs of disease (typically 24–28 days following tumor challenge), then the tumor load was assessed in the lungs. Results are expressed as mean  $\pm$  SD. The experiments presented are representative of two experiments involving six animals per groups; \*\*\*,  $p < 0.0001$ . *c*, Depletion of CD4<sup>+</sup> or CD25<sup>+</sup> cells was conducted as described above, then splenocytes prepared from spleens harvested 1 wk after the boost injection was incubated overnight with the SIINFEKL peptide. ELISPOT assay was used to measure INF- $\gamma$  secretion. The experiments presented are representative of three experiments involving three animals per groups. Results are expressed as mean  $\pm$  SD. \*\*,  $p = 0.0016$  (GK1.5 vs mAb control treatment); \*\*,  $p = 0.0023$  (PC61 vs mAb control treatment).

#### *E. coli* LLO/OVA vaccination induce high-avidity CTLs

Because OVA-specific CD8<sup>+</sup> T cell expressing the immunodominant T cell epitope OVA<sub>257–264</sub> SIINFEKL are clearly critical to the antitumor effect leading to the in vivo rejection of B16/OVA tumor (6, 26), the presence of SIINFEKL-specific CTLs was tested in mice in the prophylactic vaccination setting. To characterize the avidity of the cytotoxic responses, splenocytes from vaccinated mice were restimulated in vitro with SIINFEKL-loaded LPS-stimulated spleen cells and CTL activity was measured 6 days later using SIINFEKL-loaded RMA-S and B16/OVA cells as targets. The results are summarized in Fig. 3*a*. Splenocytes from *E. coli* LLO/OVA-vaccinated animals showed a strong response against SIINFEKL-loaded RMA-S cells and B16/OVA tumor cells (>75% killing at the highest E:T ratio), suggesting that the CTLs are of high avidity. By contrast, weak CTL responses against SIINFEKL-loaded RMA-S cells and B16/OVA tumors (<25% killing at the highest E:T ratio) were detected in splenocytes cultures from *E. coli* OVA-vaccinated mice, while no cytotoxic activity was found in splenocyte cultures from *E. coli* or *E. coli* LLO control mice. ELISPOT assay measuring INF- $\gamma$  secretion by T

cells confirmed the requirement of LLO to activate T cell function (Fig. 3*b*): only *E. coli* LLO/OVA SIINFEKL-stimulated splenocytes induced significant INF- $\gamma$  production ( $250 \pm 84$  spots/well vs  $48 \pm 8$  or  $50 \pm 16$  spots/well in *E. coli* and *E. coli* OVA-vaccinated animals, respectively;  $p = 0.004$ ).

#### *E. coli* OVA and *E. coli* LLO/OVA vaccines generate a pool of memory T cells biased toward effector responses (effector memory T cells (T<sub>EM</sub>))

In this study, we investigate the ability of the *E. coli* vaccines to program memory responses. Central memory T cells (T<sub>CM</sub>) and T<sub>EM</sub> can be differentiated by the relative expression of CD62L on the CD44<sup>high</sup> population (27, 28). We used these markers to analyze the accumulation of CD4 and CD8 T<sub>CM</sub> and T<sub>EM</sub> in the spleen and inguinal lymph nodes of vaccinated animals. Results showed that the proportions of total memory phenotype were similar between *E. coli* OVA and *E. coli* LLO/OVA. Both vaccines generated a pool of memory T cells biased toward effector responses (T<sub>EM</sub>) compared with PBS-injected mice (see Table I).

Table II. Mice received two s.c. injections of bacteria at a 1-wk interval<sup>a</sup>

	Spleens		Inguinal LNs	
	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 (%)
Naive	3.66 ± 0.66	96 ± 0.86	3.24 ± 0.5	89.57 ± 2.1
<i>E. coli</i>	4.13 ± 0.52	96.94 ± 2.6	3.26 ± 0.06	82.34 ± 2.7
<i>E. coli</i> LLO	4.17 ± 0.82	91.33 ± 2.4	3.89 ± 0.27	88.75 ± 2.06
<i>E. coli</i> OVA	3.46 ± 0.54	93.56 ± 3.7	3.37 ± 1	86.11 ± 8.6
<i>E. coli</i> LLO/OVA	4.18 ± 0.15	93.9 ± 6.7	3.12 ± 1.13	73.88 ± 9.5

<sup>a</sup> Single-cell suspensions were prepared from the spleens and inguinal LNs collected 7 days after the boost injection then stained with the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Regulatory T Cell Staining kit (eBioscience). CD4<sup>+</sup>CD25<sup>high</sup> T cell frequencies and Foxp3 expression within this population as measured by FACS analysis are shown. The data presented are representative of two separate experiments involving six animals per group.

#### Depletion of CD4<sup>+</sup> or CD25<sup>+</sup> T cells in *E. coli* OVA-vaccinated animals allows the establishment of CD8<sup>+</sup> T cell-mediated tumor-protective immune responses

To assess the relative contribution of T cell subsets in tumor protection, CD8<sup>+</sup> as well as CD4<sup>+</sup> T cells were depleted in vivo in the vaccination model. CD4<sup>+</sup> T cell depletion was conducted at the stage of T cell priming (on days 5 and 10 after the first vaccination). CD8<sup>+</sup> T cells were depleted on days 10 and 17 after bacterial injections. CD8<sup>+</sup> T cells depletion reversed the protection induced by *E. coli* LLO/OVA vaccine as well as the modest but significant protection observed upon *E. coli* OVA vaccination, demonstrating the essential role of CD8<sup>+</sup> T cells in tumor protection (Fig. 4a). CD4<sup>+</sup> T cells depletion at the stage of T cell priming had no impact on *E. coli* LLO/OVA vaccination, suggesting that CD8<sup>+</sup> T cell priming in vivo can occur in the absence of CD4<sup>+</sup> T cell help. Unexpectedly, CD4<sup>+</sup> T depletion turned *E. coli* OVA into a vaccine as effective as *E. coli* LLO/OVA suggesting that a subset of CD4<sup>+</sup> T cells inhibited the cells mediating the antitumor response. Moreover, mice receiving combined treatments with depleting Abs against CD4 and CD8 T cells showed progressive tumor growth, confirming that the antitumor activity unmasked by CD4<sup>+</sup> T cell depletion is dependent on the presence of CD8<sup>+</sup> T cells (Fig. 4a).

We next attempted to characterize the CD4<sup>+</sup> T cells subset inhibiting the CD8<sup>+</sup> T cell-mediated response. NK cells depletion with PK136 Abs allowed us to exclude the possibility of any regulation by NK or NKT cells (data not shown). CD4<sup>+</sup>CD25<sup>high</sup> Treg have been shown to enhance tumor growth by regulating cells mediating tumor immunosurveillance (29). Treg cells may therefore exert suppressor functions following *E. coli* OVA vaccination. To assess the involvement of these cells, mice were depleted of CD25<sup>+</sup> cells by i.p. administration of the PC61 mAb (anti-CD25) on day -1 before the vaccination regimen and the mice were challenged 1 wk after the boost vaccination. The tumor burden in different conditions is presented in Fig. 4b. When compared with depletion using an irrelevant mAb control (RatIgG1), CD25 depletion had no statistically significant effect on the number of tumor nodules in mice receiving *E. coli*, *E. coli* LLO, or *E. coli* LLO/OVA vaccines. By contrast, this depletion resulted in a dramatic, statistically significant reduction in the tumor load in *E. coli* OVA-vaccinated animals ( $p < 0.001$  compare with mAb control-treated mice), turning *E. coli* OVA into a vaccine as potent as *E. coli* LLO/OVA. Interestingly, IFN- $\gamma$  secretion as measured by ELISPOT analysis was restored in mice that received *E. coli* OVA vaccines and that were depleted of CD4<sup>+</sup> or CD25<sup>+</sup> cells (Fig. 4c). Splenocytes from mAb-treated control stimulated low IFN- $\gamma$  secretion ( $50 \pm 16$  spots/well) that was boosted by CD4<sup>+</sup> depletion ( $400 \pm 250$  spots/well,  $p = 0.002$ ) or by CD25<sup>+</sup> depletion ( $175 \pm 90$  spots/well,  $p = 0.001$ ) (Fig. 4c). These data strongly suggest that *E. coli* OVA vaccines induce OVA-specific CD8<sup>+</sup> T cells that

are necessary for the antitumor effect but that Treg cells prevent their expansion.

#### *E. coli* OVA and *E. coli* LLO/OVA vaccines induce similar frequencies of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells

To investigate whether LLO expression affects Treg cell expansion, we compared the prevalence of these cells in the spleen and LNs close to the site of inoculation in vaccinated animals. No significant differences in CD4<sup>+</sup>CD25<sup>high</sup> T cell frequencies were found in the spleens and inguinal LNs in all groups of mice, as assessed by FACS analysis, and Foxp3<sup>+</sup> expression within this population was similar (80–100%) (Table II). (For an example of CD4<sup>+</sup>CD25<sup>high</sup> T cells, see Fig. 5a).

To further investigate whether LLO expression in the bacterial vaccine could prevent Treg cell expansion, splenocytes from naive mice were cultured with the various *E. coli*-activated BMDCs, used as APCs, and the kinetics of CD4<sup>+</sup>CD25<sup>high</sup> T appearance were established in the cultures. We first demonstrated that the level of expression of costimulatory and MHC class II molecules on BMDCs following activation with the different bacteria were similar (data not shown). CD4<sup>+</sup>CD25<sup>high</sup> T cells arising from the cultures increased over time, however, no significant differences in the frequencies as well as in Foxp3 expression were noticeable

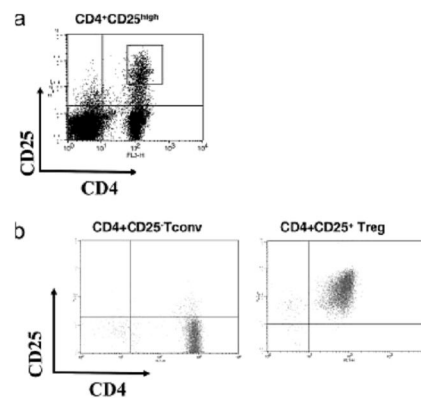


FIGURE 5. Flow cytometric analyses of CD4<sup>+</sup>CD25<sup>high</sup>, CD4<sup>+</sup>CD25<sup>-</sup>, and CD4<sup>+</sup>CD25<sup>+</sup> T cells. *a*, Representative experiment depicting CD4<sup>+</sup>CD25<sup>high</sup> gated on live lymphocytes is shown. *b*, Single-cell suspensions were obtained from the spleens of vaccinated mice. CD4<sup>+</sup> T cells were negatively selected and fractionated into CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> subsets by MACS (Miltenyi Biotec), using PE-labeled anti-CD25 mAb followed by anti-PE microbeads, according to the manufacturer's instructions. The purity of the cells was analyzed by staining with anti-CD4 and anti-CD25 Abs.

Table III. Splenocytes prepared from naive C57BL/6 mice were cultured with BMDCs that have been previously activated with *E. coli*, *E. coli* LLO, *E. coli* OVA, or *E. coli* LLO/OVA<sup>a</sup>

	Naive BMDCs		<i>E. coli</i> -Activated BMDCs		<i>E. coli</i> LLO-Activated BMDCs		<i>E. coli</i> OVA-Activated BMDCs		<i>E. coli</i> LLO/OVA-Activated BMDCs	
	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 <sup>+</sup> (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 <sup>+</sup> (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 <sup>+</sup> (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 <sup>+</sup> (%)	CD4 <sup>+</sup> CD25 <sup>+</sup> (%)	FoxP3 <sup>+</sup> (%)
Day 1	1.57 ± 0.2	75 ± 5	2.19 ± 0.3	65 ± 9	2.07 ± 0.5	66 ± 8	2.44 ± 0.2	62 ± 4	1.74 ± 0.5	74 ± 3
Day 2	2.41 ± 0.6	91 ± 3	2.56 ± 0.05	88 ± 8	2.46 ± 0.5	88 ± 8	2.66 ± 0.3	82 ± 4	2.44 ± 0.1	88 ± 8
Day 3	7.02 ± 0.3	87 ± 6	7.96 ± 0.7	87 ± 8	7.46 ± 0.9	89 ± 9	8.03 ± 0.9	86 ± 3	5.47 ± 2.1	85 ± 5

<sup>a</sup> Cells were harvested at different time points then stained for CD4, CD25, and Foxp3 expression by using the eBioscience Treg Staining kit before being analyzed by FACS. The experiments presented are representative of two separate experiments.

between groups (Table III). From these results, we concluded that LLO expression in the vaccine does not affect Treg expansion.

#### CD4<sup>+</sup>CD25<sup>+</sup> isolated from *E. coli* OVA- or *E. coli* LLO/OVA-vaccinated mice induce different levels of suppression

To test their functionality, Treg were purified after the vaccination regimen and yielded a CD4<sup>+</sup>CD25<sup>+</sup> population that was >90% pure (Fig. 5b). We performed titration studies with different amounts of Treg mixed with their corresponding CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup> responders (conventional T cells (T<sub>CONV</sub>)) (10<sup>5</sup>/assay). Cultures were stimulated with an anti-CD3 mAb. Treg cells from each of the vaccine systems did not proliferate upon TCR stimulation (data not shown). In the MLR, T<sub>CONV</sub> proliferation from *E. coli* or *E. coli* OVA-vaccinated animals was gradually reduced according to Treg dosing, and >50% inhibition was observed at a Treg/T<sub>CONV</sub> ratio of 1:1 (Fig. 6a). By contrast, inhibition of proliferation of responders was dramatically reduced with Treg cells from *E. coli* LLO or *E. coli* LLO/OVA-vaccinated mice (<20% at

the highest ratio). Importantly, Treg cells from the *E. coli* OVA vaccine system inhibited the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup> responders from the *E. coli* LLO/OVA vaccine system as effectively as they inhibited their corresponding responders (Fig. 6b). Thus, animals receiving *E. coli* LLO or *E. coli* LLO/OVA vaccines had overall reduced regulatory functions compared with mice receiving *E. coli* or *E. coli* OVA vaccines, suggesting that LLO expression in the *E. coli* vaccines reduces/suppresses Treg cell function.

#### *E. coli* LLO/OVA vaccine stimulate CD8-mediated responses

Finally, we analyzed the cytokine response induced by the different vaccines in the sera, in SIINFEKL-stimulated splenocytes cultures, and in the various *E. coli* activated-BMDCs cultures. Results are summarized in Table IV. IL-2, IL-4, IL-10, and IFN- $\gamma$  serum levels were just above the threshold of detection and not significantly different between any of the animals given the *E. coli* vaccines. IFN- $\gamma$  levels were significantly lower in splenocyte

FIGURE 6. CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from *E. coli* OVA or *E. coli* LLO/OVA-vaccinated animals induce different levels of suppression. a, Mice received two s.c. injections of *E. coli*, *E. coli* LLO, *E. coli* OVA, or *E. coli* LLO/OVA and splenocytes prepared from spleens harvested 7 days after the boost injection were separated into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. In a MLR, CD4<sup>+</sup>CD25<sup>high</sup> (variable number) were cultured with CD4<sup>+</sup>CD25<sup>-</sup> (10<sup>5</sup>) T cells for 4 days in the presence of 0.5  $\mu$ g/ml anti-CD3 and 2  $\times$  10<sup>5</sup> naive irradiated splenocytes. Proliferation of responders was measured by adding [<sup>3</sup>H]thymidine for the last 18 h of culture period and inhibition of proliferation was determined. The experiments presented are representative of three independent experiments. \*\*\*, *p* < 0.001 *E. coli* vs *E. coli* LLO and *E. coli* OVA or *E. coli* LLO/OVA. b, Mice received two s.c. injections of *E. coli* OVA or *E. coli* LLO/OVA and splenocytes prepared from spleens harvested 7 days after the boost injection were separated into CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. CD8<sup>+</sup> T cells were also purified from *E. coli* LLO/OVA-vaccinated animals. In a MLR, CD4<sup>+</sup>CD25<sup>+</sup> (variable numbers) from *E. coli* OVA were cultured with 10<sup>5</sup> of their corresponding responders (CD4<sup>+</sup>CD25<sup>-</sup> T cells) or responders from *E. coli* LLO/OVA-vaccinated animals (CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup> T cells) for 4 days in the presence of 0.5  $\mu$ g/ml anti-CD3 and 2  $\times$  10<sup>5</sup> naive irradiated splenocytes. Proliferation of responders was measured by adding [<sup>3</sup>H]thymidine for the last 18 h of culture period and inhibition of proliferation was determined. The experiments presented are representative of two independent experiments.

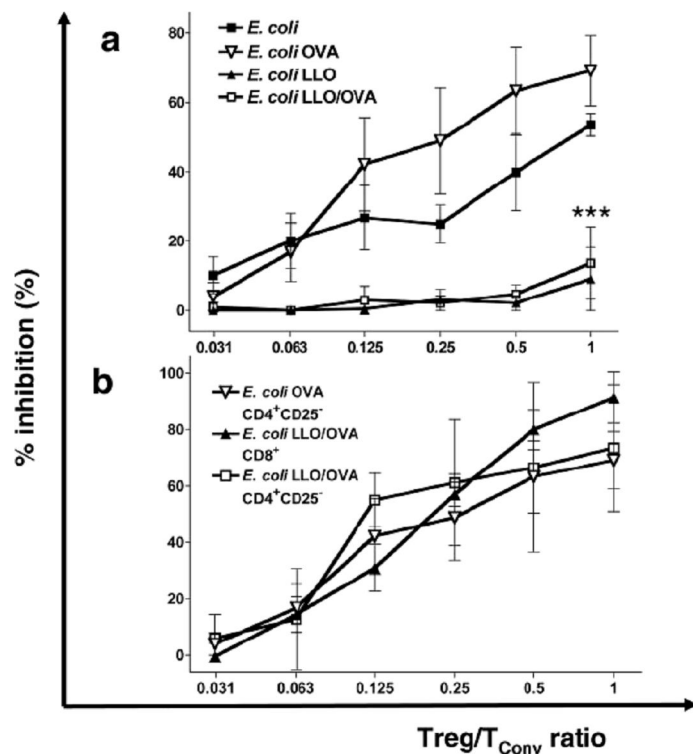


Table IV. Cytokine production upon bacterial vaccination

	IL-4	IL-10	IFN- $\gamma$	IL-2
Serum levels (pg/ml) <sup>a</sup>				
Naive	0.8	7.9	4.7 (0–9.4)	7.6 (0–15.6)
<i>E. coli</i>	0.7 (0–3.5)	9 (2.8–24.8)	5 (0.5–9.7)	24 (0–45)
<i>E. coli</i> LLO	0	7.5 (3.3–10.8)	14 (6.4–19.7)	12.8 (0–40)
<i>E. coli</i> OVA	11 (0–36)	4.5 (3.3–5.4)	11.5 (5.9–13)	11 (0–17)
<i>E. coli</i> LLO/OVA	0	15.3 (4.2–34.8)	3.9 (0–9.7)	15.2 (0–44)
BMDCs cultures (pg/ml) <sup>b</sup>				
Naive	7.9 $\pm$ 0.5	10 $\pm$ 3.5	5 $\pm$ 2	14 $\pm$ 5
<i>E. coli</i>	6.2 $\pm$ 1.1	17 $\pm$ 13	7 $\pm$ 1.3	15 $\pm$ 2.2
<i>E. coli</i> LLO	6.2 $\pm$ 0.9	25 $\pm$ 12	20 $\pm$ 6.2	17 $\pm$ 2.5
<i>E. coli</i> OVA	6.4 $\pm$ 0.25	140 $\pm$ 21	5 $\pm$ 1.1	15 $\pm$ 6.5
<i>E. coli</i> LLO/OVA	6.5 $\pm$ 1.3	44 $\pm$ 12	40 $\pm$ 9***	16 $\pm$ 5.3
Splenocyte cultures (pg/ml) <sup>c</sup>				
	IL-4		IL-10	
	Unstimulated	Stimulated	Unstimulated	Stimulated
Naive	12 $\pm$ 2.2	9.2 $\pm$ 2.1	11.2 $\pm$ 2.6	9.6 $\pm$ 4.2
<i>E. coli</i>	9.2 $\pm$ 3.1	9.1 $\pm$ 3.5	8.8 $\pm$ 5.4	12.3 $\pm$ 4.1
<i>E. coli</i> LLO	7.5 $\pm$ 0.5	8.5 $\pm$ 0.1	8.8 $\pm$ 1.2	9.8 $\pm$ 2.5
<i>E. coli</i> OVA	8 $\pm$ 2.5	9.6 $\pm$ 2.3	10.6 $\pm$ 3.3	10.8 $\pm$ 4.2
<i>E. coli</i> LLO/OVA	9.8 $\pm$ 6.3	12.5 $\pm$ 1.2	8 $\pm$ 2.3	11 $\pm$ 3.5
	IFN- $\gamma$		IL-2	
	Unstimulated	Stimulated	Unstimulated	Stimulated
Naive	152.3 $\pm$ 13.2	172.3 $\pm$ 9.6	30.2 $\pm$ 11	31.9 $\pm$ 13
<i>E. coli</i>	208 $\pm$ 22.3	332 $\pm$ 30	28.3 $\pm$ 6.5	20.4 $\pm$ 10.3
<i>E. coli</i> LLO	140 $\pm$ 46.4	170 $\pm$ 14	23.9 $\pm$ 5.9	20.2 $\pm$ 3.6
<i>E. coli</i> OVA	176 $\pm$ 37	450 $\pm$ 14.2	22.2 $\pm$ 3.2	26.7 $\pm$ 6.2
<i>E. coli</i> LLO/OVA	126 $\pm$ 52	841 $\pm$ 102***	21 $\pm$ 8.7	25.7 $\pm$ 1.4

<sup>a</sup> Mice received two injections doses of  $10^8$  of *E. coli*, *E. coli* LLO, *E. coli* OVA, or *E. coli* LLO/OVA at a 1-wk interval. Sera were collected 1 wk after the boost injection and were analyzed for cytokine production by ELISA. The experiments presented are representative of two separate experiments involving six animals per group.

<sup>b</sup> BMDCs were preactivated with the different bacteria as described in *Materials and Methods* then cultured for 48 h before being assayed for cytokine production. The experiments presented are representative of two separate experiments. \*\*\*,  $p < 0.001$  *E. coli* OVA vs. *E. coli* LLO/OVA.

<sup>c</sup> Mice received two injections doses of  $10^8$  of *E. coli*, *E. coli* LLO, *E. coli* OVA, or *E. coli* LLO/OVA at a 1-wk interval. Single-cell suspensions were prepared from the spleens collected 1 wk after the boost injection. Splenocytes were left unstimulated or were restimulated in vitro for 3 days with the SIINFEKL peptide before being assayed for cytokine production. The experiments presented are representative of two separate experiments involving three animals per group. \*\*\*,  $p < 0.001$  *E. coli* OVA vs. *E. coli* LLO/OVA.

cultures from animals that received the *E. coli* OVA vaccines as compare with animals that received *E. coli* LLO/OVA vaccines ( $450 \pm 14$  vs  $841 \pm 102$  pg/ml). The same trend was found in BMDC cultures that were activated by *E. coli* and *E. coli* OVA as compare with BMDCs cultures that were activated by *E. coli* LLO and *E. coli* LLO/OVA, while the inverse correlation was observed for IL-10 production. Altogether, these data support the conclusion that the response to *E. coli* LLO/OVA vaccines is CD8 mediated.

## Discussion

To our knowledge, this is the first report to demonstrate that the presence of LLO in a vaccine formulation suppresses Treg cell function. Using the model tumor Ag OVA, we have demonstrated that *E. coli* LLO/OVA is a powerful tool for successful antitumor vaccination, through its ability to generate high-avidity CTL and Treg functional defect leading to the rejection of tumor. Recombinant *E. coli* have already been described as protein delivery vectors for professional phagocytic cells (6, 20, 21) and presentation of the OVA epitope on MHC class I by *E. coli* LLO/OVA has been shown to be orders of magnitude more efficient than *E. coli* OVA strains (6, 20). Therefore, the difference in efficacy of the two vaccines may be attributed to a difference in efficiency of MHC class I presentation on APCs.

This hypothesis is partly confirmed by the observation that higher frequencies of OVA-specific CD8<sup>+</sup> T cells were found in *E. coli* LLO/OVA-vaccinated animals. However, additional data demonstrated that the removal of CD4<sup>+</sup> or CD25<sup>+</sup> T cells allowed the activation of OVA-specific CD8<sup>+</sup> T cells in *E. coli* OVA-immunized animals resulting in tumor rejection. Thus, the *E. coli* OVA vaccines activate OVA-specific CD8 responses but Treg cells prevent their expansion, and coexpression of LLO enhances the effectiveness of the vaccine through the inhibition of Treg cells.

In a previous report (5), a recombinant *Listeria monocytogenes* strain that expresses and secretes the human papilloma virus E7 protein fused to a nonhemolytic form of LLO (Lm-LLO-E7) was shown to be effective against established E7-expressing tumors. Interestingly, no protection was observed with a strain that expresses and secretes E7 alone, not fused to LLO, (Lm-E7) and depletion of CD4<sup>+</sup> or CD25<sup>+</sup> cells turned Lm-E7 into an effective treatment (5). The authors found increased numbers of CD4<sup>+</sup>CD25<sup>high</sup> T cells in Lm-E7-vaccinated mice compared with Lm-LLO-E7-immunized animals and no difference in Treg cell function (30). The design and modes of action of these bacterial vaccines may explain these differences. In our study, we used an *E. coli* strain of bacteria, a Gram-negative, while *Listeria*, a Gram-positive bacterium was used for the

other study. In addition, recombinant *Listeria* are likely to reach the cytosol intact and will secrete either E7 and LLO-E7 proteins, while *E. coli* LLO/OVA LLO perforates the lysosomal membrane and allows the release of the bacterial contents in the cytosol (LLO is not fused to the Ag and lacks its secretion signal sequence).

In mice, the elimination of CD4<sup>+</sup> suppressor T cells, using various strategies has been largely reported to enhance antitumor immunity (31–36). Many studies have reported elevated levels of CD4<sup>+</sup>CD25<sup>+</sup> T cells in patients with different types of cancers (37–41). Similarly, greater disease burden and poorer overall survival are correlated to increased Treg cells (1, 39, 42, 43). These observations have led to the development of new therapeutic strategies aiming at the elimination of Treg cells in cancer patients and, so far, a single clinical trial has been reported, involving an IL-2/diphtheria toxin conjugate to target CD25 at the surface of Tregs (44). In this context, the recombinant *E. coli* LLO-expressing tumor Ags provides a unique system by inducing specific cytotoxic responses and selectively inhibiting regulatory T cell function. However, it is still unexplained how LLO inhibits Treg cell function and studies are currently underway to determine the exact mechanism of this inhibition.

The *E. coli* vaccination system offers a number of advantages. First, the bacteria are nonpathogenic and the delivery of Ag relies on the lysis of the bacteria by APCs. The paraformaldehyde fixation kills the bacteria while retaining the antitumor activity, providing an additional safety feature. Furthermore, as the full-length cDNA of the Ag is expressed in the bacteria, the vaccine is unlikely to be restricted to a specific HLA haplotype in humans. Current work in our laboratory in the context of tumor Ags that are self Ags has demonstrated that vaccination with recombinant *E. coli* LLO expressing the Wilm's tumor 1 Ag (WT-1) (45, 46) led to a significant control of WT-1-expressing tumors in C57BL/6J mice, correlated to specific CTL responses and Treg functional defect (J. Nitcheu-Tefit, M. S. Dai, and G. Vassaux, unpublished observations). Based on these results, it is possible to envisage the use of this system in humans.

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## Disclosures

The authors have no financial conflict of interest.

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