Chapter Ⅰ

Literature Review

Ⅰ.1 Introduction

Ⅰ.1. 1 The immune system

We live in a world full of infectious and non-infectious microbes, including viruses, bacteria, fungi, protozoa and multicellular parasites. These microbes would have killed us if there were not the immune system in our bodies to protect us from the invasion of these microbes. Our body mainly has three lines of protection. The first line is the exterior defense, such as skin, lysozyme in tears, mucus in bronchi, rapid pH change in the gut, and so on. For those very few infectious agents that can pass through all these physical and biological barriers, there are second and third lines of protection, i.e. the innate immune response and adaptive immune response. The innate immune response can protect the body from pathogen invasion non-specifically. Phagocytic cells, such as monocytes, macrophages and polymorphonuclear neutrophils circulate in the blood and migrate into tissue to bind to microbes, internalize them and kill them. The innate immune response is very important because not only does it non-specifically destroy infectious microbes, but it also may present antigens to the adaptive immune system, the third line of protection. The core of the adaptive immune system is lymphocyte cells, which can specifically recognize individual pathogens and destroy them. According to their different origins and functions, lymphocytes are categorized into two groups: T lymphocytes (T cells) and B lymphocytes (B cells). In adult mammals, although all lymphocytes are derived from bone marrow, T cells develop in the thymus while B cells mature in the bone marrow. Upon infection by pathogens, B cells secret pathogen-specific antibodies to neutralize the extracellular pathogens and trigger antibody-mediated responses, while some types of T cells recognize the cells infected by intracellular pathogens and destroy them. Rather than being two separate systems, innate immunity and adaptive immunity interact with each other. The antibodies secreted from B cells and cytokines from T cells can activate phagocytic cells, while phagocytic cells present antigens to activate T cells (Fig. Ⅰ.1). The special feature of the adaptive immune response is that it has memory, which is the basis of immunoprophylaxis (Male, 2001).

Ⅰ.1. 2 Vaccines

Vaccine is an important contribution by the field of immunology to human healthcare. More than 200 years ago, Dr. Edward Jenner developed the first vaccine for smallpox. This epidemic disease has been eradicated from the population by global application of smallpox vaccine. Traditional vaccines are killed or attenuated pathogens which can induce strong protective immune responses to the relevant diseases. These vaccines have proven to be functional for protective immunity. However,

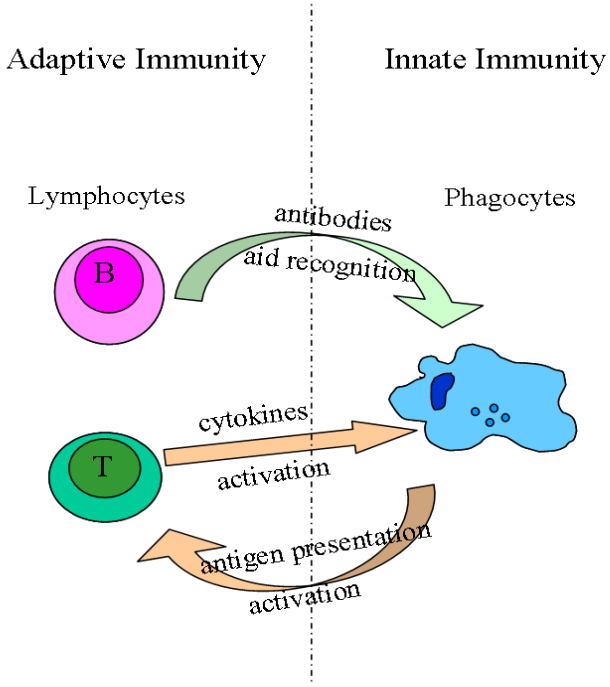


Fig.I. 1 The innate immunity interacts with the adaptive immunity. Antibodies secreted from B cells and cytokines from T cells (adaptive immunity) activate phagocytes (innate immunity) while phagocytes activate T cells by presenting antigens.

one of the drawbacks of traditional vaccines is the residual of virulence, which may cause health hazards to susceptible populations.

Through the development of new biotechnology techniques, antigenic molecules can either be purified from pathogens or produced in recombinant expression systems by using genetic engineering technology. Many antigenic molecules, due to their size and composition, are poor immunogens. However, when these molecules are administered with an immune-stimulating entity (e.g. adjuvant), measurable immune responses are observed. Thus, typical subunit vaccines usually include antigenic molecules mixed with adjuvants. To design a good vaccine, there are three factors that need to be considered: (1) its ability to trigger protective immune responses (2) its safety and (3) its cost (Beverley, 2001). The immune response to a particular pathogen/antigen is regulated by many factors, including antigen, antibodies, complements (blood proteins whose action "complements" the work of antibodies), antigen-presenting cells (APC), B lymphocytes, T lymphocytes, and even the nervous system (Cooke, 2001). Among these, T lymphocyte regulation is the most important for vaccine design.

Ⅰ.1. 3 Regulation of the immune response by T lymphocytes

Ⅰ.1. 3.1 T helper cells

Lymphocytes developing from the thymus are called T cells. A group of T cells that play a central role in immune response regulation, called T helper (Th, CD4+) cells, have a unique function, in that they respond to antigens presented by antigen presenting cells (APC) and initiate immune responses. There are two major subtypes of T helper cells distinguished by the profile of cytokines that are secreted: T helper cells type 1 (Th1) and T helper cells type 2 (Th2). Th1 cells mainly secrete interleukin-2 (IL-2), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12) and interleukin-18 (IL-18). The principal function of Th1 cells is to enhance phagocyte-mediated defense against infections (cell-mediated immunity). Th2 cells mainly secrete interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10) and interleukin-13 (IL-13). Th2 cells are excellent helpers for B lymphocytes and stimulate the production of high levels of IgM and non-complement-fixing IgG isotypes (antibody-mediated immunity), which is very important in protection against large, extracellular parasite/pathogen invasion. Th1 and Th2 cells develop from the same T-cell precursor. The differentiation may be influenced by the manner and environment in which the precursors are stimulated (reviewed by Abbas et al., 1996; Spellberg et al., 2001; Fig. Ⅰ.2).

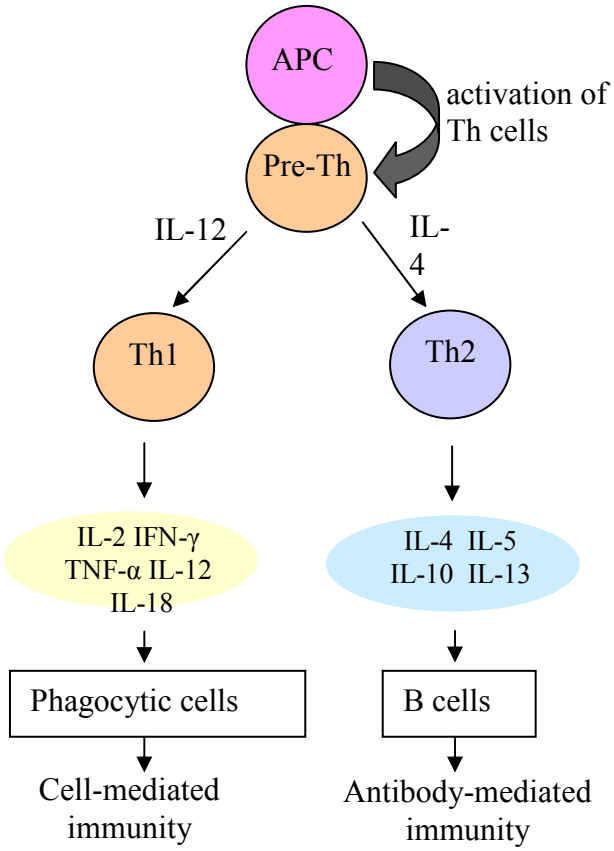


Fig.I.2 Antigen presenting cells (APC) activate precursor T cells to differentiate into Th1 or Th2, depending on the manner and environment in which the precursors are stimulated. As an important immunomodulator, IL-12 stimulates the differentiation of Th1 cells and elicits cell-mediated immunity. APC: dendritic cells; Th: T helper cells

Ⅰ.1. 3. 2 Th1/Th2

Many investigators believe that the homeostasis of Th1 and Th2 cells is critical to the immune system regulation. Studies on mice infected with the intracellular protozoan *Leishimania* were first used to demonstrate that the resistance and susceptibility to this infectious disease are related to the antimicrobial Th1 and Th2 responses, respectively, i.e., the association of Th1 cell development with the control of infection and Th2 cell growth with the progressive disease (Reiner et al., 1995). Further investigations found that resistance to intracellular pathogens or microbes that can not be phagocytosed is usually associated with the induction of Th1 immune response. Some large, extracellular pathogens, like helminthes, induce Th2 immunities, but the role of the predominant Th2 immune response in resistance of infection is still disputed (Spellberg et al., 2001). Investigators believe that the immune system uses Th1 responses for protection against acute infection and switches to Th2 responses when the danger is passed in order to reestablish homeostasis and protect the host from autoinflammatory destruction (Spellberg et al., 2001). Imbalance of Th1/Th2 is involved in a wide range of pathological processes. Overreaction of Th1 cells may cause autoimmune disorders while overreaction of Th2 cells is involved with allergic diseases (reviewed by Abbas et al., 1996; Spellberg et al., 2001).

It is generally believed that appropriate type of immune responses elicited by a vaccine is crucial to its efficiency. Vaccines inducing Th1 immunity have been proven highly effective at preventing diseases, whereas vaccines predominantly inducing Th2 immunity increase susceptibility to infectious agents, especially intracellular and encapsulated pathogens (Sin et al., 1999; Buchanan et al., 2001; Cheers et al., 1999; Sharma et al., 1996; Winter et al., 2005). Th1-biased immune responses are thought to be critical for the control of many pathogen infections and should be induced by vaccines directed against intracellular pathogens to obtain protective immunities. On the contrary, for extracellular pathogen vaccines, observations suggest it is better to induce Th2-directed immunities to stimulate the production of antibodies to neutralize invading pathogens (reviewed by Abbas et al., 1996; Spellberg et al., 2001).

The maturation of Th1/Th2 cells are influenced by many dynamic factors, including antigen dose, nature of the antigen, route of administration, cell-to-cell interaction with APC (antigen-presenting cells), the diversity and relative intensity of these interactions, and the cytokine receptors available on the naïve cell (Kidd, 2003). Many cytokines, secreted by lymphocytes, can be used as therapeutics and adjuvants in vaccines to skew the immune responses toward a Th1 or Th2 immune output. IL-12, one of the cytokines secreted by Th1 cells, is an ideal potential adjuvant for eliciting the proliferation of Th1 cells and enhancing Th1-biased immune responses. Many investigations utilizing

IL-12 as an adjuvant to elicit Th1 immune responses have showed great potential (Buchanan et al., 2001; Lynch et al., 2003; Salem et al., 2004). In my research, plant-derived IL-12 was explored for its application as a mucosal immune modulator in directing Th1-biased immune responses for utility in developing effective anti-cancer and viral vaccines.

Ⅰ.2 Interleukin-12 (IL-12)

Ⅰ.2.1 Discovery of IL-12

Interleukin 12 (IL-12), also called natural killer cell stimulatory factor and cytotoxic lymphocyte maturation factor, was isolated and identified from supernatants of phytohemagglutinin (PHA)-activated human peripheral blood mononuclear cells (PBMC) and Epstein-Barr virus (EBV)-transformed human B lymphoblastoid cells by two independent research groups (Wong et al., 1988; Kobayashi et al., 1989). It is a disulphide-linked heterodimer consisting of a 35kDa alpha subunit (p35) and a 40kDa beta subunit (p40) (Gubler et al., 1991; Wolf et al., 1991). IL-12 is mainly produced *in vivo* by T helper cells type 1 (Th1), phagocytic cells, B cells and other antigen-presenting cells, and it induces the production of interferon-gamma (IFN-γ) in T and natural killer cells. Because IL-12 plays important roles in the innate resistance to infections, the generation of Th1 cells and the differentiation of cytotoxic T lymphocytes (CTL), it is considered a key cytokine in the communication between innate resistance and adaptive immunity (Trinchieri, 1994; 2003).

Ⅰ.2.2 Potential applications of IL-12

Because of its key immunostimulatory function, IL-12 has been studied as a potential therapeutic agent for a variety of diseases, including cancer and viral infection. Studies have shown that IL-12 has anti-tumor activity in many mouse models. Tumor-specific CTLs producing recombinant IL-12 enhanced the production of Th1 cytokines and also promoted the survival rate of mice bearing these tumors by countering the adverse Th2 cytokine environment produced by the tumor cells. This suggests that IL-12 may contribute to a better adoptive immunotherapy (Wagner et al., 2004). IL-12-expressing oncolytic herpes virus, when administrated to mice with disseminated pulmonary metastatic squamous cell carcinoma (SCC), significantly enhanced survival (Wong et al., 2004). Many clinical trials have been performed to evaluate the anti-tumor activity of IL-12 (Table Ⅰ.1). Most of them did not progress beyond Phase II due to toxicity issues and, in some cases, associated deaths (Leonard et al., 1997). Toxicity associated with systemic administration of IL-12, a problem with many cytokines, remains a major hurdle for its application in healthcare, which may be

overcome by alternative delivery routes such as mucosal or paracrine delivery (reviewed by Salem et al., 2006).

Table Ⅰ.1: Human clinical trials of IL-12 as anti-tumor therapeutics

Disease	Phase	Delivery route and dosage	Results	References
Renal cancer, melanoma or colon caner	I	Intravenous administration, 500 ng/kg as maximum	Dose-limited toxicity	Atkins et al., 1997
Advanced renal cell cancer	II	Intravenous administration, 500 ng/kg	Severe toxicity (two deaths)	Leonard et al., 1997
Metastatic melanoma	Pilot study	Subcutaneous administration, 500 ng/kg	Well tolerated and anti-tumor activity indicated	Bajetta et al., 1998
Advanced renal cell cancer	I	Subcutaneous administration, 1250 ng/kg maximum	Dose-limited toxicity	Portielje et al., 1999
Advanced renal cell cancer	I	Intravenous administration, 1000 ng/kg	Dose-limited toxicity, significant immunological activity	Robertson et al., 1999
Advanced malignancies	I	Subcutaneous administration, 300 ng/kg maximum	Tolerated	Ohno et al., 2000
Advanced malignant melanoma	I	Subcutaneous or intravenous administration, 100 ng/kg maximum	Tolerated	Cebon et al., 2003
Metastatic renal cell carcinoma or malignant melanoma	I	Subcutaneous administration, 500 ng/kg as maximum	Phase II dosage recommended	Alatrash et al., 2004
Head and neck squamous cell carcinoma	I	Intratumoral administration, 300 ng/kg	Dose-limited toxicity, measurable immunological responses	van Herpen et al., 2004

IL-12 may also be utilized as a potential therapy for allergies associated with Th2-biased immune responses. Recent studies have demonstrated that IL-12 can inhibit allergen-induced airway hyperresponsiveness, eosinophilia, inflammation, and Th-2 cytokine expression in mice (Bruselle et al., 1997; Gavett et al., 1995; Hofstra et al., 1998; reviewed by Leonard et al., 2003).

IL-12 shows great potential as a therapeutic for many diseases. However, over-active IL-12 is involved in autoimmune diseases and graft-resistance in transplantation. An IL-12 antagonist may be

utilized as a therapeutic for autoimmune diseases and graft-resistance in transplantation to inhibit the activity of IL-12, resulting in less active Th1 cells and CTLs. IL-12 was linked with deterioration of the health condition of the mice adoptively transferred with experimental autoimmune encephalitis, which can be reduced with IL-12 antibody treatments (Leonard et al., 1996). Treatment of mice with anti-IL-12 monoclonal antibody (mAb) also promoted graft survival in liver allograft, supposedly due to IL-12 antagonism enhancing apoptosis of alloreactive T cells (Li et al., 2001).

Another important application of IL-12 is to use it as an adjuvant for viral and cancer vaccines to enhance protective Th1-biased immune responses, for example, in vaccines targeting streptococcus pneumoniae and neisseria meningitides (Buchanan et al., 2001; Lynch et al., 2003). These will be discussed in detail in another part of this chapter.

Ⅰ.2.2.1 Interleukin-12 and toxicity

Although IL-12 is a crucial cytokine and potentially has multiple therapeutic applications, clinical trials have shown significant toxicity from IL-12 administered intravenously. In a Phase II clinical trial, two renal cell cancer patients died from side-effects of systemic administration of IL-12. It was observed that the toxic shock syndrome of IL-12 was associated with high levels of IFN-γ in the blood that, at least in part, mediated this toxicity (Leonard et al., 1997). A single dose of IL-12 as a pretreatment before the consecutive dosing attenuated IL-12-induced IFN-γ production and reduced toxicity by unknown mechanisms (Leonard et al., 1997).

While this observed toxicity of IL-12 has precluded its current use in clinical practice, researchers have attempted to find alternative approaches for safer and more efficacious delivery of IL-12. Tumor-specific antibodies have been investigated as fusion partners to specifically target IL-12 to tumor tissue. Mouse *in vivo* studies have shown that antibody-fused IL-12 retains both antibody specificity and anti-tumor activity, which may provide a viable alternative to systemic administration of IL-12 (Peng et al., 2001). More recent studies have also illustrated that mucosal delivery of IL-12 was as much, or maybe even more efficient than systemic administration of IL-12 with significantly reduced toxicity (reviewed by Salem et al., 2006). Intranasal delivery of IL-12 into mice exhibited enhanced T cell activation and Th1-associated immunoglobulin (IgG_{2a}) levels, but induced less systemic IFN-γ production than observed following subcutaneous injection of IL-12. This suggests that intranasal delivery of IL-12 is efficacious but not as toxic as systemic administration (Huber et al., 2003). Salem et al. (2004) developed a gel matrix (F2 gel) that has the capability for sustained local delivery of IL-12. Compared to systemic administration, this paracrine administration of IL-12

showed a much lower systemic level of IL-12 in serum but with enhanced functional immune responses, indicating that paracrine release of IL-12 may be a feasible alternative for administration of IL-12. All these alternative delivery methods may reduce the side-effects of IL-12 in clinical practice.

Ⅰ.2.2.2 Interleukin-12 and mucosal adjuvants

IL-12 may also be used as a mucosal adjuvant. Much evidence has shown that mucosal vaccines (nasal, oral, dermal, etc) can not only induce systemic immune responses accompanied with high secretion of IgG in the blood, but can also enhance the secretion of immunoglobulin A (IgA) from mucosal lymphoid tissue (reviewed by Freytag and Clements, 2005). Mucosal IgAs show advantages in preventing the attachments of pathogens and removal of pathogens from mucosal tissue. Moreover, mucosal lymphoid tissues also contain specialized epithelial cells called M cells that can take up antigens and present them efficiently to antigen presenting cells (APCs), initiating immune responses (Fig. Ⅰ.3). As mentioned before, most subunit antigens are not immunogenic enough, so a mucosal adjuvant has to be added to trigger strong immune responses. Investigations have shown that protective antigens with the addition of mucosal adjuvants elicited not only mucosal immune responses but also systemic immune responses (Hou et al., 2002; Jones et al., 2003).

Many macromolecules have been investigated as mucosal adjuvants, although it is still not very clear how adjuvants function to enhance the immune response. Yuki and Kiyono classified mucosal adjuvants into two categories: mucosal immunostimulatory adjuvants (cholera toxin, enterotoxin, cytokines, CpG oligodeoxynucleotides, saponin, etc.) and mucosal vaccine delivery vehicles (liposome, Bacillus Calmette Guerin (BCG) adjuvant, edible vaccine). The most studied mucosal adjuvants are cholera toxin (CT) and enterotoxin (LT), but there is little chance for them to be approved for human vaccine use because of their toxicity. Efforts have been made to develop detoxified CT and LT which may have potential in clinic application of mucosal vaccines (Freytag and Clements, 1999; Yuki and Kiyono, 2003).

As a strong immunomodulator, IL-12 has been investigated as a potential mucosal adjuvant to direct Th1-biased immune responses. In mice, IL-12 enhanced the antigen-specific immune responses and increased survival rates when challenged with infectious pathogens (Arulanandam et al., 2001; Lynch et al., 2003). IL-12, together with cholera toxin subunit B (CTB), synergistically enhanced both systemic and local mucosal antibody responses to HIV antigens (Albu et al., 2003). IL-12 as an adjuvant has also been investigated in human clinical trials. IL-12, co-administered with peripheral blood mononuclear cell (PBMC) pulsed with antigen, induced antigen-specific immune responses and

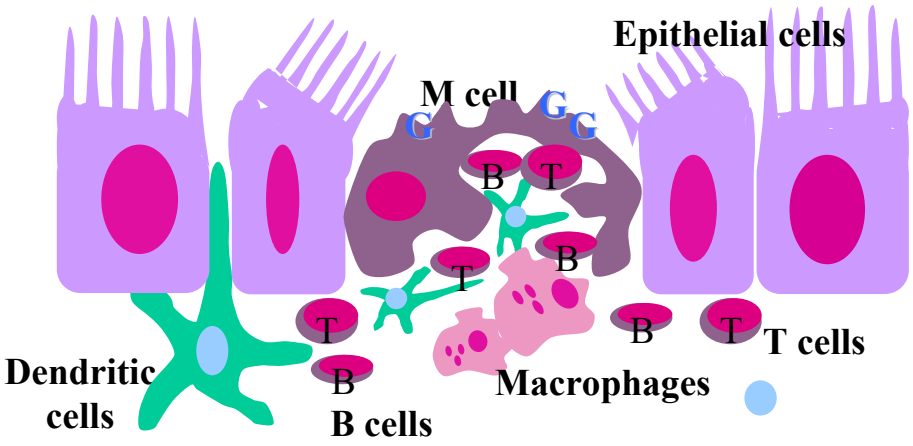


Fig.I.3 The structure of mucosal-associated lymphoid tissue (MALT) (Courtesy of Dr. M. Dolan).

showed clinical activity (Gajewski et al., 2001). Although many clinical trials supported the adjuvanticity of IL-12, there are some controversies between results from mice studies and those from human clinical trials. Buchanan et al. (2001) demonstrated that IL-12 significantly enhanced humoral immune responses in mice to pneumococcal and meningococcal conjugated vaccines and increased protection against bacterial challenge. However, IL-12, when co-administered with pneumococcal polysaccharide vaccine (PPV) to healthy volunteers, induced numerically higher antigen specific IgG, but the difference was not significant (Hedlund et al., 2001). Thus, IL-12 may elicit host-specific differences when used as adjuvant for extracelluar pathogen vaccines (Villinger, 2003)

Ⅰ.2.3 Recombinant expression of IL-12

Ⅰ.2.3.1 IL-12 protein structure

IL-12 is a complex heterodimeric glycoprotein with subunits associated through disulfide interaction. From human cells it was first found that both subunits of IL-12 are glycosylated and encoded by distinct genes located on different chromosomes (Gubler et al., 1991; Wolf et al., 1991). Schoenhaut et al. (1992) cloned the murine IL-12 genes and found 70% sequence identity at the amino acid level between human and mouse p40 subunits and 60% identity between human and mouse p35 subunits. It has been shown that human IL-12 is unable to stimulate the proliferation of mouse lymphocytes at low concentration, while murine IL-12 is active on both mouse and human lymphocytes (Schoenhaut et al., 1992).

The amino acid sequence of p35 shows homology to class 1 cytokines IL-6 and granulocyte-colony stimulating factor (Merberg et al., 1992). The p40 subunit is not homologous to any known cytokine, but its primary sequence shows similarity to the hematopoietic cytokine receptor family, such as the extracellular domains of IL-6 receptor α chain (IL-6Rα) and the ciliary neutrotrophic factor receptor (Gearing and Cosman, 1991). The crystallogram of human IL-12 has confirmed that p70 is formed by the 1:1 complex of p35 and p40 and resembles a soluble class 1 cytokine-receptor complex. These two subunits are linked together by two inter-strand disulfide bonds and a unique interlocking topography formed by charged residues. The interaction of these charged residues from p35 and p40 is very critical to p70 formation, as the protein will assemble in the absence of disulfide bonds. However, the disulfide bond may ensure the stable association and stoichiometry of these two subunits (Yoon et al., 2000)

The p40 subunit was shown to be a specific antagonist of IL-12 heterodimer in mice (Mattner et al., 1993). Moreover, p40 associates not only with p35 forming IL-12, but also with another

molecule, p19, to form a new heterodimeric cytokine IL-23 (Oppmann et al., 2000). As a result, the stoichiometry of p40 and p35 is essential in recombinant expression of IL-12.

Ⅰ.2.3.2 Recombinant expression of IL-12

As the level of IL-12 expressed in natural sources is very limited, many investigators have explored the possibility of recombinant expression. Since IL-12 is encoded by two separate genes, various methods have been developed to co-express these two genes and assemble them into a bioactive heterodimer, including cotransfection with two plasmids, transfection with a single plasmid with two expression cassettes in tandem array, retrovirus vectors containing internal ribosome entry site (IRES) sequences to express two subunits from a single polycistronic transcript, or insertion of subunit cDNAs into recombinant adenoviral vector region 1 (E1) and region 3 (E3) respectively (Tahara et al., 1994, 1995; Rakhmilevich et al., 1996; Bramson et al., 1996). Because of the antagonism of p40 in mice and complexity of recombinant expression, several research groups have also developed single chain IL-12 constructs by linking two subunits with a 15-amino acid linker $(Gly_4Ser)_3$, showing advantages in stoichiometry and preventing the production of excess free p40 (Anderson et al., 1997; Lieschke et al., 1997). Single-chain IL-12 (scIL-12) produced in yeast and mammalian cells stimulated the secretion of IFN-γ from peripheral blood mononuclear cells (PBMC) and enhanced the proliferation of pre-activated PBMC, thus demonstrating full bioactivity (Foss et al., 1999; Lieschke et al., 1997).

Recombinant IL-12 has been expressed in three different plant bioproduction systems: tobacco cell suspension culture (Kwon et al., 2003), transgenic tobacco (Gutierrez-Ortega et al., 2004) and transgenic tomato (Gutierrez-Ortega et al., 2005). Tobacco cell suspension cultures expressing human IL-12 (hIL-12) were established from plants that were derived from sexual crossing of plants expressing each subunit of the protein. The accumulation of secreted hIL-12 in the media could reach as high as 175μg/L at day 5, but thereafter this protein degraded quickly, probably due to the presence of proteases in the media. IL-12 from plant cell culture showed bioactivity in enhancing the proliferation of phytohemagglutinin (PHA) activated peripheral blood mononuclear cells (Kwon et al., 2003). Gutierrez-Ortega et al. generated transgenic tobacco expressing single chain human IL-12 (schIL-12, 2004) and transgenic tomato expressing single chain murine IL-12 (scmIL-12, 2005). The expression level of scmIL-12 from tomato (~7 μg/g of fresh weight) is much higher than that of schIL-12 from tobacco (~40 ng/g of fresh weight). The difference of schIL-12 and scmIL-12 expression level in plants might be protein-specific, although human IL-12 and murine IL-12 are highly homologous in amino-acid sequence. Crude extracts of transgenic plants expressing IL-12 showed some bioactivity as

they induced the secretion of IFN-gamma from lymphocytes. However, they failed to demonstrate activity in stimulating the proliferation of lymphocytes because non-transgenic control also elicited high level of cell proliferation (Gutierrez-Ortega et al., 2004; 2005) (Table Ⅰ.2).

Table Ⅰ.2: Comparison of plant-derived IL-12 from literature

IL-12 resources	Mouse IL-12	Human IL-12	Human IL-12
Production system	*Lycopersicon esculentum* cv. Tanksley	*Nicotiana tabacum* cv. Xantii	*Nicotiana tabacum L* cv. Havana cell culture
Constructs	Single chain	Single chain	Sexual crossing from plants expressing each subunit
Promoter	CaMV35S	Double-enhanced CaMV 35S	CaMV 35S
Signal peptide	Endogenous	Never mentioned, endogenous assumed	Endogenous
IL-12 expression level	7.3 μg/g of leaf fresh weight	40 ng/g of leaf fresh weight	Peak at 175μg/L at day 5; Drops to 40μg/l after day11
Extraction buffer	10 mM Na_2HPO_4, 3 mM KH_2PO_4, 100 mM NaCl, 25 mM sodium ascorbate	10 mM Na_2HPO_4, 3 mM KH_2PO_4, 100 mM NaCl, 25 mM sodium ascorbate	N.A.
Bioactivity	Induced the secretion of IFN-γ from T lymphocytes	Induced the secretion of IFN-γ from human NK cells	Induced the proliferation of human PBMC
References	Gutierrez-Ortega et al., 2005	Gutierrez-Ortega et al., 2004	Kwon et al., 2003

It is generally believed that only eukaryotic expression systems can produce bioactive IL-12. However, one research group reported biologically active single chain IL-12 produced by the gram-positive bacterium *Lactococcus. lactis* (Bermudez-Humaran et al., 2003).

Because IL-12 has great potential as a therapeutic, there is significant demand for IL-12 for research purposes and clinical trials. Presently there are two sources of commercialized reagent-grade human and murine IL-12, i.e. insect cells (*Sf*21) and mammalian cells (CHO, Chinese hamster ovarian cells). These two production systems require high standards of sterilization and costly fermentation facilities, which makes the price of IL-12 quite expensive and hinders IL-12 potential application in research and clinical use. Moreover, currently there is no source of clinical IL-12 (produced under

cGMP conditions). Transgenic plants may provide a cost-effective and scalable bioproduction system for valuable pharmaceuticals, like IL-12. This will be further discussed later in this chapter.

Ⅰ.3 Ricin B –a molecular carrier

Clinical applications of IL-12 are limited by two significant factors: 1) lack of a cost-effective, scalable bioproduction platform for clinical-grade IL-12 and 2) issues of IL-12 toxicity that emerged during clinical trials associated with dose and systemic administration. Our laboratory has been involved in studies to understand and exploit the lectin binding activities of the galactose/galactosamine-binding subunit of ricin (RTB) to deliver proteins to immune responsive cells through mucosal surfaces. Although the focus of these experiments have been to test the efficacy of RTB in delivering and presenting vaccine antigens (Medina-Bolivar et al., 2003), we reasoned that RTB may also facilitate the delivery of IL-12 to the immune system, thus avoiding systemic administration and potentially reducing dose requirements- the key elements linked with IL-12 toxicity. Because my research includes the development and testing of IL-12:RTB fusions, I have provided background information on ricin and RTB below.

Ⅰ.3.1 Ricin

Ricin, a type II ribosome-inactivating protein (RIP) toxin from castor bean (*Ricinus communis*), is a heterodimer consisting of two subunits, ricin A (RTA) and ricin B (RTB). The toxin subunit, RTA has RNA *N*-glycosidase activity and modifies 28S rRNA in eukaryotic ribosomes, thus inhibiting protein synthesis and causing toxicity (Endo et al., 1987). RTB is the non-toxic, carbohydrate-binding subunit that binds to the glycans on cell surfaces so that ricin is internalized by receptor-mediated endocytosis (Mohanraj et al., 1995). Ricin is a useful protein for cellular protein transport studies. Ricin participates in multiple subcellular transport pathways following endocytosis (Sandvig et al., 2000). Ricin is internalized into cells through either the clathrin-coated pits or non-coated pits pathway (Magnusson et al., 1993). Indirect evidence suggests that ricin reaches the endoplasmic reticulum (ER) where RTB is degraded and RTA is translocated to the cytosol by translocons (ER-located Sec61; Roberts and Smith, 2004). Ricin may also be transported from endosomes to lysosomes and become digested (Magnusson et al., 1993), or recycled to the cell surface by transcytosis (van Deurs et al., 1990).

Ricin B (RTB), the carbohydrate-binding subunit, plays an important role in the process of endocytosis and transcytosis of ricin. The two galactose-binding domains in RTB are essential for RTA delivery, not only in cell surface binding but also in intracellular trafficking (Newton et al., 1992).

As ricin lacks the ER retention sequence KDEL, it is believed that RTB interacts with ER-localized chaperones, such as calreticulin, so that ricin is transported retrograde into the ER (reviewed by Roberts and Smith, 2004). As a glycoprotein with mannose-terminated glycans, RTB has also been shown to enter cells via clathrin-coated pits by binding to the D-mannose receptor (Frankel et al., 1997).

Ⅰ.3.2 Ricin B (RTB) as a potential molecular carrier/mucosal adjuvant

The fact that RTB is involved in multiple transport pathways in the cell suggests that RTB may be utilized as a molecular carrier to facilitate the presentation of antigens to the immune responsive cells (Fig. Ⅰ.4). It has been demonstrated that RTB can carry covalently linked proteins into cultured mammalian cells (Reidy and Cramer, unpublished). Disarmed ricin fused with a small peptide facilitated the presentation of the small peptide to major histocompatibility complex (MHC) class I molecules, indicating disarmed ricin could be an adjuvant for cancer vaccines (Smith et al., 2002). RTB has also been produced as a fusion partner in transgenic plants to carry antigens (Medina-Bolivar et al., 2003; Choi et al., 2006). Medina-Bolivar et al. (2003) found that transgenic tobacco-produced RTB functioned as a mucosal adjuvant and mediated the induction of primarily Th2-skewed immune responses to its fusion partner green florescent protein (GFP).

We are interested in determining whether RTB may aid in the targeted delivery of IL-12. IL-12 demonstrated toxicity in humans when administered systemically (Leonard et al., 1997). Localized presentation through subcutaneous gels or intranasal administration has suggested that “targeted delivery” may allow for the desired IL-12 efficacy at much lower concentrations, thus greatly reducing toxicity (Huber et al., 2003; Salem et al., 2004). The advantage of mucosal delivery of IL-12 encouraged us to explore the potential of RTB as a vector to carry IL-12 specifically and efficiently into mucosal sites, to facilitate the presentation of IL-12 to immune systems.

Ⅰ.4 Transgenic plants as promising bioproduction systems for pharmaceutical proteins

Ⅰ.4.1 Plant-based vaccines

Transgenic plants have been proposed as promising vaccine production and delivery systems due to some advantages plants have over other vaccine production systems (Streatfield et al., 2001). Vaccines (antigens and adjuvants) and other valuable proteins can be produced in plants by utilizing genetic engineering technology. Mucosal vaccines, usually protective antigens from pathogens and/or mucosal adjuvants, can be expressed in transgenic plants and then be delivered to animals and humans

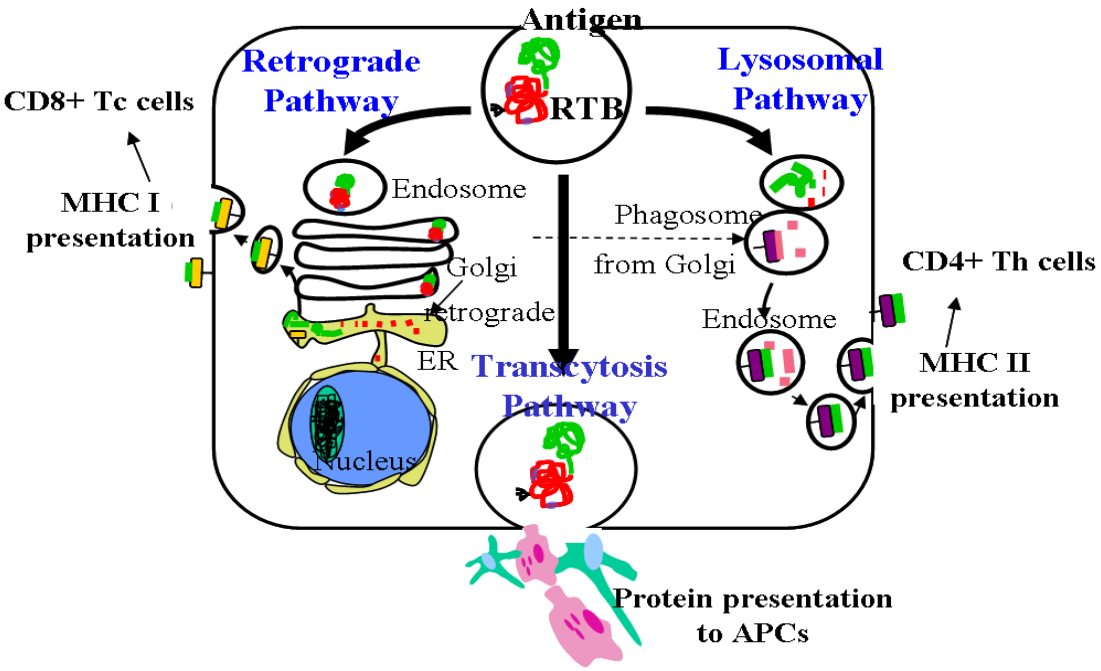


Fig.I. 4 Multiple transport pathways for ricin B (RTB) trafficking in cells. The lysosomal pathway may facilitate antigen presentation to T helper (Th) cells via major histocompatibility complex (MHC) II molecules; the retrograde pathway to the ER may mediate the presentation of antigen to cytotoxic T cells (Tc) via MHC I molecules; and the transcytosic pathway may enhance antigen delivery to antigen presenting cells (APCs). This figure was kindly provided by Dr. M. Dolan with minor modification.

by several different strategies. All the investigations on production of mucosal vaccines in transgenic plants can be divided in two general strategies. One approach is to express mucosal vaccines in transgenic crops and administer the vaccine orally with minimal processing of the plant material (edible vaccine). The second strategy is to express protective antigens in the plant system with subsequent purification prior to mucosal administration.

Clinical trials demonstrated that food crops expressing bacterial or viral antigens, when fed to volunteers, elicited antigen-specific systemic immune responses and mucosal immune responses (Webster et al., 2002; Thanavala et al., 2005). Though edible vaccines have great potential, there are several hurdles that challenge the development of edible vaccines, including issues of oral tolerance, food allergies, and dosage control.

Both IL-12 and IL-12:RTB fusion may be important for these vaccine strategies because of their ability to specifically modulate immune responses. This may be particularly important for plant-based vaccines where delivery through the oral route is being proposed. The incorporation of a immunomodulator like IL-12 into edible vaccines as an adjuvant may help in preventing oral tolerance and possible food allergies as IL-12 elicits Th1-enhanced immune responses.

Ⅰ.4.1.1 Edible vaccines

Many crop species have shown the ability to produce edible vaccines that induced protective immunity to many types of pathogens (See Table Ⅰ.3). As a popular food source, potato has been studied as a possible candidate for edible vaccines. Potato tubers expressing test antigens have been fed to mice with or without adjuvants and analyzed for the pathogen-specific IgG and IgA in serum, feces, saliva and/or urine in vaccinated mice. The results suggest that transgenic potato is able to produce edible vaccines, but with different antigens the immune responses induced by these edible potato vaccines vary. Human papillomavirus (HPV) capsid protein L1 forms highly immunogenic virus-like particles (VLPs) and demonstrated great potential in controlling HPV-associated diseases. Warzecha et al. (2003) showed that HPV type 11 capsid protein L1 expressed in transgenic potato formed VLP structures. Mice immunized with these transgenic potato tubers exhibited similar anti-VLP immune responses to those induced by purified insect cell-derived VLP administered parenterally (Warzecha et al., 2003). Mucosal adjuvants (CT, LT) are likely to enhance the immunogenicity of antigens (Warzecha et al., 2003; Mason et al., 1996; Wu, et al., 2003). These results suggest that transgenic potato has great potential to be used as edible vaccines.

Table Ⅰ.3: Plant-based edible vaccine studies

Vaccine	antigen	Adjuvant	Plant host	Immune response	reference
Human papillomavirus type 16	capsid protein L1	None	Potato	Weak immune response	Biemelt et al., 2003
Human papillomavirus type 11	capsid protein L1	LT	Potato	Potentially protective immune response	Warzecha et al., 2003
Enterotoxin	Enterotoxin B subunit	None	Potato	Elicits systemic and local immune response on parentally primed mice	Lauterslager et al., 2001
Norwalk virus	Capsid protein	CT	Potato	Elicits systemic response, but weak mucosal response	Mason et al., 1996
Rotavirus	VP7	CT/CTB	Potato	Systemic &mucosal response	Wu, et al., 2003
Respiratory syncytial virus	F protein	None	Tomato	Systemic & mucosal response	Sandhu et al., 2000
Enterotoxin	LT-B	None	Maize	Systemic & mucosal response	Chikwamba et al., 2002
Measles	hemagglutinin glycoprotein	None	Carrot	Systemic response with neutralizing activity	Marquet-Blouin et al., 2003
foot and mouth disease virus	VP1	None	Alfalfa	Virus specific response	Wigdorovitz et al., 1999

Besides potato, transgenic tomato, maize, carrot and alfalfa are also possible targets for edible vaccines. Several subunit vaccines have been produced in transgenic tomato (Jani et al., 2002; Ma et al., 2003; Sandhu et al., 2000; Walmsley et al., 2003). Transgenic tomatoes expressing respiratory syncytial virus-F protein were used to orally immunize mice and induced protective Th1 type cell immune responses (Sandhu et al., 2000). Maize-derived LT-B and CT-B induced protective immune responses against enterotoxin and cholera toxin in mice following oral administration (Chikwamba et al., 2002). Carrots, which can be eaten raw by humans, have been used to produce measles virus H protein. Immunization of mice with transgenic carrots induced the secretion of measles virus-specific neutralizing IgG antibody (Marquet-Blouin et al., 2003). Foot and mouth virus VP1 produced in

transgenic alfalfa was shown to induce protective immunity when fed to mice (Wigdorovitz et al., 1999). Thus, it appears that food crops are capable of producing functional edible vaccines and hold great potential as a cost-effective vaccine platform.

Tobacco, which can easily be transformed, is also considered to be a favorite target for plant-based vaccine investigations, even though it is not considered "edible". It is often used as a model system to study the possibility of producing active subunit vaccines in plants as the first step to edible vaccines. A protective antigen from anthrax has been produced in transgenic tobacco and shown to be bioactive (Aziz et al., 2002). An antigenic protein from swine oedema has been produced in tobacco seeds as a model for edible vaccines (Rossi et al., 2003).

Although the concept of edible vaccines is very promising, there are several questions that edible vaccine research should address before it can be effectively applied to human healthcare. First, the expression level of edible vaccines in transgenic crops must be stable and sufficiently consistent to meet quality control requirements for dose quantification. Oral vaccines are likely to require higher antigen amounts compared to other mucosal routes due to loss of antigen by digestion in the alimentary canal. Second, edible vaccines should be able to induce strong and stable protective immune responses. Addition of proper adjuvants and immunomodulators to edible vaccines may be required to elicit strong and lasting immune responses, but currently there are no protein-based mucosal adjuvants approved for human healthcare. This dims the potential of edible vaccines. Another hurdle of edible vaccines is the issue of oral tolerance. However, a recent publication displays that oral tolerance can be avoided by the addition of adjuvants, such as saponins and other immune stimulating complexes (ISCOMS) (Mowat, 2005).

Ⅰ.4.1.2 Plant as production platform for vaccine components

Edible vaccines are under investigation by many research groups. However, plants may also be effective bioproduction systems for mucosal vaccines administered by other mucosal routes including: intranasal, dermal, rectal, genital as well as other delivery routes to mucosal tissue. While these strategies generally require partial or extensive purification which significantly impacts cost compared to edible strategies, plants are still advantageous in vaccine production in that they are cost-effective, safe and able to produce complex proteins.

Subunit vaccines are generally not good immunogens and the addition of an adjuvant is necessary to induce protective immune responses. Cholera toxin (CT) and enterotoxin (LT) are not only potential vaccines for intestinal infectious diseases, but are also considered to be strong mucosal

adjuvants. While their toxicity prohibits their application in human healthcare, their non-toxic binding B subunits (CTB, LTB) may be sufficient in targeting the antigen specifically to mucosal tissue and facilitating antigen presentation to the immune system. Some researchers have transformed plants with CTB or LTB genes and produced these recombinant non-toxic proteins as immunogens and adjuvants (Wagner et al., 2004). Under the same concept, some other plant lectins were also produced in transgenic plants for potential use as mucosal adjuvant, like mistletoe lectin (Lavelle et al., 2004) and the non-toxic subunit B of ricin (Medina-Bolivar et al., 2003).

In January 2006, a biotechnology company (Dow AgroSciences LLC, Indianapolis, IN, company website, 2006) announced that it received the world's first regulatory approval of a plant-made vaccine from the United States Department of Agriculture (USDA) Center for Veterinary Biologics. A tobacco cell based bioproduction system was utilized for production of this chicken Newcastle disease vaccine. This is a milestone not only for this company but also for the plant-based vaccine industry.

Ⅰ.4.2 Plant-based production of pharmaceutical proteins

Besides vaccines, plants have also been utilized to produce other valuable pharmaceutical proteins, such as serum proteins, monoclonal antibodies, cytokines, and lysosomal enzymes. Plants have several advantages in producing pharmaceutical proteins: (1) ability to process complex protein, (2) lack of human or animal infectious agents, (3) cost effective, and (4) ease to scale up. Although plants have been proven to produce bioactive pharmaceutical proteins, their possible application in healthcare is limited by several issues. The most critical one is that the plant shows a slight difference in post-translational modification from animals. This difference may affect proteins' activity and pharmacokinetics *in vivo*. It could also be immunogenic to the animal's immune system and trigger some severe problems. However, these difference in plant post-translational modification in plants can be addressed through genetic engineering strategies, making the concept of plant-produced pharmaceutical proteins quite feasible (reviewed by Cramer et al., 1999).

Ⅰ.5 Significance and objectives of the research

As a very important immunomodulator, IL-12 has demonstrated great potential in tumor therapy and anti-cancer and anti-viral vaccines (reviewed by Trinchieri, 1994; 2003). However, the clinical application of IL-12 is limited by two factors: (1) lack of large-scale of production and (2) possible toxicity. Plants, which have successfully produced many pharmaceutical proteins, may provide a valuable production system for IL-12. Because human IL-12 lacks the ability to stimulate

mouse lymphocyte cells, I chose to initially produce murine IL-12 so that its activity could be investigated in a mouse model. Therefore, the overall objectives of this research were to produce murine IL-12 (mIL-12) and mIL-12:RTB fusions in transgenic tobacco and characterize the resulting transgenic products.

The research was designed to address the following questions: a) Can plants synthesize functional IL-12 and accumulate this complex heterodimer glycoprotein? b) Does plant-derived IL-12 show equivalent biological activities as animal-cell-derived IL-12 in *in vitro* cell assays and *in vivo* mouse vaccine trials? c) Can plants produce IL-12 as an RTB-fusion protein and does the product retain both RTB and IL-12 bioactivity? and d) Does RTB enhance IL-12 delivery to cultured mammalian cells?

The first objective was to develop transgenic plants for expression of mIL-12 and mIL-12:RTB fusions. DNA sequences encoding the single chain form of mIL-12 were fused to the constitutive promoter 35S. Multiple RTB fusions were generated such that RTB was either on the N-terminus (RTB:IL-12) or C-terminus (IL-12:RTB) of mIL-12. All constructs were transferred into *Nicotiana tabacum* cv Xanthi by *Agrobacterium*-mediated transformation, and transformants were screened to identify strong expressers. Southern hybridization was used to verify that transgenes were integrated into tobacco genome and to assess copy number.

The second objective was to characterize and purify transgenic products. IL-12 p70 ELISAs were used to quantify IL-12 yields and western analyses were used to assess size and conformation of the recombinant proteins. The lectin activity of RTB fusions were assessed by asialofetuin-binding assay. Protein purification strategies were developed for plant-derived mIL-12 and mIL-12:RTB fusions.

The third objective was to test IL-12 bioactivity *in vitro*. Following purification, plant-derived mIL-12 and mIL-12:RTB were compared to animal-cell-derived IL-12 for their ability to enhance the proliferation of mouse splenocytes and stimulate the secretion of IFN-gamma.

The fourth objective was to test RTB activity *in vitro*. Purified mIL-12:RTB fusion was investigated in a drug transport assay model using cultured mammalian cells to assess the ability of RTB to facilitate the uptake of IL-12 into certain type of cells.

The fifth objective was to assess IL-12 activity *in vivo*. The effect of plant-derived mIL-12 on immune responses was studied following intranasal vaccination of mice. Key indicators of Th1

immunity versus Th2 immunity were assessed to demonstrate plant-derived mIL-12 could enhance Th1 immune responses.

These experiments will determine the feasibility of plants to serve as an efficient and scalable production platform for this complex and medically important cytokine. Thus this research could have significant implications for the medical arena because of IL-12's value as an anti-cancer therapeutic and as a Th1-biased vaccine adjuvant for both cancer and infectious disease medications.

Ⅰ.6 References

Dow AgroSciences Achieves World's First Registration for Plant-Made Vaccines. Dow AgroSciences, January 31, 2006, www.dowagro.com/newsroom/corporatenews/2006/20060131b.htm.

Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996; 383: 787-793.

Alatrash G, Hutson TE, Molto L et al. Clinical and immunologic effects of subcutaneously administered interleukin-12 and interferon alfa-2b: phase I trial of patients with metastatic renal cell carcinoma or malignant melanoma. J.Clin.Oncol. 2004; 22: 2891-2900.

Albu DI, Jones-Trower A, Woron AM, Stellrecht K, Broder CC, Metzger DW. Intranasal vaccination using interleukin-12 and cholera toxin subunit B as adjuvants to enhance mucosal and systemic immunity to human immunodeficiency virus type 1 glycoproteins. J.Virol. 2003; 77: 5589-5597.

Anderson R, Macdonald I, Corbett T, Hacking G, Lowdell MW, Prentice HG. Construction and biological characterization of an interleukin-12 fusion protein (Flexi-12): delivery to acute myeloid leukemic blasts using adeno-associated virus. Hum.Gene Ther. 1997; 8: 1125-1135.

Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against Streptococcus pneumoniae infection. Infect.Immun. 2001; 69: 6718-6724.

Atkins MB, Robertson MJ, Gordon M et al. Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies. Clin.Cancer Res. 1997; 3: 409-417.

Aziz MA, Singh S, Anand KP, Bhatnagar R. Expression of protective antigen in transgenic plants: a step towards edible vaccine against anthrax. Biochem.Biophys.Res.Commun. 2002; 299: 345-351.

Bajetta E, Del Vecchio M, Mortarini R et al. Pilot study of subcutaneous recombinant human interleukin 12 in metastatic melanoma. Clin.Cancer Res. 1998; 4: 75-85.

Bermudez-Humaran LG, Langella P, Cortes-Perez NG et al. Intranasal immunization with recombinant Lactococcus lactis secreting murine interleukin-12 enhances antigen-specific Th1 cytokine production. Infect.Immun. 2003; 71: 1887-1896.

Beverley P. Vaccination. In: Ivan Roitt, Jonathan Brostoff, David Male, editors. Immunology. Mosby, 2001.

Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Muller M. Production of human papillomavirus type 16 virus-like particles in transgenic plants. J.Virol. 2003; 77: 9211-9220.

Bramson J, Hitt M, Gallichan WS, Rosenthal KL, Gauldie J, Graham FL. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: *in vitro* and *in vivo* production of biologically active interleukin-12. Hum.Gene Ther. 1996; 7: 333-342.

Bruselle GG, Kips JC, Peleman RA et al. Role of IFN-gamma in the inhibition of the allergic airway inflammation caused by IL-12. Am.J.Respir.Cell Mol.Biol. 1997; 17: 767-771.

Buchanan RM, Briles DE, Arulanandam BP, Westerink MA, Raeder RH, Metzger DW. IL-12-mediated increases in protection elicited by pneumococcal and meningococcal conjugate vaccines. Vaccine 2001; 19: 2020-2028.

Cebon J, Jager E, Shackleton MJ et al. Two phase I studies of low dose recombinant human IL-12 with Melan-A and influenza peptides in subjects with advanced malignant melanoma. Cancer Immun. 2003; 3: 7.

Cheers C, Janas M, Ramsay A, Ramshaw I. Use of recombinant viruses to deliver cytokines influencing the course of experimental bacterial infection. Immunol.Cell Biol. 1999; 77: 324-330.

Chikwamba R, Cunnick J, Hathaway D, McMurray J, Mason H, Wang K. A functional antigen in a practical crop: LT-B producing maize protects mice against Escherichia coli heat labile enterotoxin (LT) and cholera toxin (CT). Transgenic Res. 2002; 11: 479-493.

Choi NW, Estes MK, Langridge WH. Synthesis of a ricin toxin B subunit-rotavirus VP7 fusion protein in potato. Mol.Biotechnol. 2006; 32: 117-128.

Cooke A. Regulation of the immune response. In: Ivan Roitt, Jonathan Brostoff, David Male, editors. Immunology. Mosby, 2001.

Cramer CL, Boothe JG, Oishi KK. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr.Top.Microbiol.Immunol. 1999; 240: 95-118.

Endo Y, Tsurugi K. RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J.Biol.Chem. 1987; 262: 8128-8130.

Foss DL, Moody MD, Murphy KP, Jr., Pazmany C, Zilliox MJ, Murtaugh MP. *In vitro* and *in vivo* bioactivity of single-chain interleukin-12. Scand.J.Immunol. 1999; 50: 596-604.

Frankel AE, Fu T, Burbage C et al. Lectin-deficient ricin toxin intoxicates cells bearing the D-mannose receptor. Carbohydr.Res. 1997; 300: 251-258.

Freytag LC, Clements JD. Bacterial toxins as mucosal adjuvants. Curr.Top.Microbiol.Immunol. 1999; 236: 215-236.

Freytag LC, Clements JD. Mucosal adjuvants. Vaccine 2005; 23: 1804-1813.

Gajewski TF, Fallarino F, Ashikari A, Sherman M. Immunization of HLA-A2+ melanoma patients with MAGE-3 or MelanA peptide-pulsed autologous peripheral blood mononuclear cells plus recombinant human interleukin 12. Clin.Cancer Res. 2001; 7: 895s-901s.

Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. J.Exp.Med. 1995; 182: 1527-1536.

Gearing DP, Cosman D. Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor. Cell 1991; 66: 9-10.

Gubler U, Chua AO, Schoenhaut DS et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc.Natl.Acad.Sci.U.S.A 1991; 88: 4143-4147.

Gutierrez-Ortega A, Avila-Moreno F, Saucedo-Arias LJ, Sanchez-Torres C, Gomez-Lim MA. Expression of a single-chain human interleukin-12 gene in transgenic tobacco plants and functional studies. Biotechnol.Bioeng. 2004; 85: 734-740.

Gutierrez-Ortega A, Sandoval-Montes C, Olivera-Flores TJ, Santos-Argumedo L, Gomez-Lim MA. Expression of functional interleukin-12 from mouse in transgenic tomato plants. Transgenic Res. 2005; 14: 877-885.

Hedlund J, Langer B, Konradsen HB, Ortqvist A. Negligible adjuvant effect for antibody responses and frequent adverse events associated with IL-12 treatment in humans vaccinated with pneumococcal polysaccharide. Vaccine 2001; 20: 164-169.

Hofstra CL, Van A, I, Hofman G, Kool M, Nijkamp FP, Van Oosterhout AJ. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsiveness, eosinophilia, and serum IgE levels. J.Immunol. 1998; 161: 5054-5060.

Hou Y, Hu WG, Hirano T, Gu XX. A new intra-NALT route elicits mucosal and systemic immunity against Moraxella catarrhalis in a mouse challenge model. Vaccine 2002; 20: 2375-2381.

Huber VC, Arulanandam BP, Arnaboldi PM *et al* . Delivery of IL-12 intranasally leads to reduced IL-12-mediated toxicity. Int.Immunopharmacol. 2003; 3: 801-809.

Jani D, Meena LS, Rizwan-ul-Haq QM, Singh Y, Sharma AK, Tyagi AK. Expression of cholera toxin B subunit in transgenic tomato plants. Transgenic Res. 2002; 11: 447-454.

Jones T, Allard F, Cyr SL et al. A nasal Proteosome influenza vaccine containing baculovirus-derived hemagglutinin induces protective mucosal and systemic immunity. Vaccine 2003; 21: 3706-3712.

Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern.Med.Rev. 2003; 8: 223-246.

Kobayashi M, Fitz L, Ryan M et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J.Exp.Med. 1989; 170: 827-845.

Kwon TH, Seo JE, Kim J, Lee JH, Jang YS, Yang MS. Expression and secretion of the heterodimeric protein interleukin-12 in plant cell suspension culture. Biotechnol.Bioeng. 2003; 81: 870-875.

Lauterslager TG, Florack DE, van der Wal TJ et al. Oral immunisation of naive and primed animals with transgenic potato tubers expressing LT-B. Vaccine 2001; 19: 2749-2755.

Lavelle EC, Grant G, Pfuller U, O'Hagan DT. Immunological implications of the use of plant lectins for drug and vaccine targeting to the gastrointestinal tract. J.Drug Target 2004; 12: 89-95.

Leonard JP, Waldburger KE, Goldman SJ. Regulation of experimental autoimmune encephalomyelitis by interleukin-12. Ann.N.Y.Acad.Sci. 1996; 795: 216-226.

Leonard JP, Sherman ML, Fisher GL et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood 1997; 90: 2541-2548.

Leonard P, Sur S. Interleukin-12: potential role in asthma therapy. BioDrugs. 2003; 17: 1-7.

Li W, Lu L, Wang Z et al. Il-12 antagonism enhances apoptotic death of T cells within hepatic allografts from Flt3 ligand-treated donors and promotes graft acceptance. J.Immunol. 2001; 166: 5619-5628.

Lieschke GJ, Rao PK, Gately MK, Mulligan RC. Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity *in vivo*. Nat.Biotechnol. 1997; 15: 35-40.

Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. Infect.Immun. 2003; 71: 4780-4788.

Ma Y, Lin SQ, Gao Y et al. Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products. World J.Gastroenterol. 2003; 9: 2211-2215.

Magnusson S, Kjeken R, Berg T. Characterization of two distinct pathways of endocytosis of ricin by rat liver endothelial cells. Exp.Cell Res. 1993; 205: 118-125.

Male D. Introduction to the immune system. In: Ivan Roitt, Jonathan Brostoff, David Male, editors. Immunology. Mosby, 2001.

Marquet-Blouin E, Bouche FB, Steinmetz A, Muller CP. Neutralizing immunogenicity of transgenic carrot (Daucus carota L.)-derived measles virus hemagglutinin. Plant Mol.Biol. 2003; 51: 459-469.

Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc.Natl.Acad.Sci.U.S.A 1996; 93: 5335-5340.

Mattner F, Fischer S, Guckes S et al. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. Eur.J.Immunol. 1993; 23: 2202-2208.

Medina-Bolivar F, Wright R, Funk V et al. A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine 2003; 21: 997-1005.

Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family. Immunol.Today 1992; 13: 77-78.

Mohanraj D, Ramakrishnan S. Cytotoxic effects of ricin without an interchain disulfide bond: genetic modification and chemical crosslinking studies. Biochim.Biophys.Acta 1995; 1243: 399-406.

Mowat AM. Dendritic cells and immune responses to orally administered antigens. Vaccine 2005; 23: 1797-1799.

Newton DL, Wales R, Richardson PT et al. Cell surface and intracellular functions for ricin galactose binding. J.Biol.Chem. 1992; 267: 11917-11922.

Ohno R, Yamaguchi Y, Toge T et al. A dose-escalation and pharmacokinetic study of subcutaneously administered recombinant human interleukin 12 and its biological effects in Japanese patients with advanced malignancies. Clin.Cancer Res. 2000; 6: 2661-2669.

Oppmann B, Lesley R, Blom B et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000; 13: 715-725.

Peng LS, Penichet ML, Dela Cruz JS, Sampogna SL, Morrison SL. Mechanism of antitumor activity of a single-chain interleukin-12 IgG3 antibody fusion protein (mscIL-12.her2.IgG3). J.Interferon Cytokine Res. 2001; 21: 709-720.

Portielje JE, Kruit WH, Schuler M et al. Phase I study of subcutaneously administered recombinant human interleukin 12 in patients with advanced renal cell cancer. Clin.Cancer Res. 1999; 5: 3983-3989.

Rakhmilevich AL, Turner J, Ford MJ et al. Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. Proc.Natl.Acad.Sci.U.S.A 1996; 93: 6291-6296.

Reiner SL, Locksley RM. The regulation of immunity to Leishmania major. Annu.Rev.Immunol. 1995; 13: 151-177.

Roberts LM, Smith DC. Ricin: the endoplasmic reticulum connection. Toxicon 2004; 44: 469-472.

Robertson MJ, Cameron C, Atkins MB et al. Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. Clin.Cancer Res. 1999; 5: 9-16.

Rossi L, Baldi A, Dell'Orto V, Fogher C. Antigenic recombinant proteins expressed in tobacco seeds as a model for edible vaccines against swine oedema. Vet.Res.Commun. 2003; 27 Suppl 1: 659-661.

Salem ML, Kadima AN, Zhou Y et al. Paracrine release of IL-12 stimulates IFN-gamma production and dramatically enhances the antigen-specific T cell response after vaccination with a novel peptide-based cancer vaccine. J.Immunol. 2004; 172: 5159-5167.

Salem ML, Gillanders WE, Kadima AN et al. Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. J.Interferon Cytokine Res. 2006; 26: 593-608.

Sandhu JS, Krasnyanski SF, Domier LL, Korban SS, Osadjan MD, Buetow DE. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. Transgenic Res. 2000; 9: 127-135.

Sandvig K, van Deurs B. Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. EMBO J. 2000; 19: 5943-5950.

Schoenhaut DS, Chua AO, Wolitzky AG et al. Cloning and expression of murine IL-12. J.Immunol. 1992; 148: 3433-3440.

Sharma DP, Ramsay AJ, Maguire DJ, Rolph MS, Ramshaw IA. Interleukin-4 mediates down regulation of antiviral cytokine expression and cytotoxic T-lymphocyte responses and exacerbates vaccinia virus infection *in vivo*. J.Virol. 1996; 70: 7103-7107.

Sin JI, Kim JJ, Arnold RL et al. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell-mediated protective immunity against herpes simplex virus-2 challenge. J.Immunol. 1999; 162: 2912-2921.

Smith DC, Gallimore A, Jones E et al. Exogenous peptides delivered by ricin require processing by signal peptidase for transporter associated with antigen processing-independent MHC class I-restricted presentation. J.Immunol. 2002; 169: 99-107.

Spellberg B, Edwards JE, Jr. Type 1/Type 2 immunity in infectious diseases. Clin.Infect.Dis. 2001; 32: 76-102.

Streatfield SJ, Jilka JM, Hood EE et al. Plant-based vaccines: unique advantages. Vaccine 2001; 19: 2742-2748.

Tahara H, Zeh HJ, III, Storkus WJ et al. Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma *in vivo*. Cancer Res. 1994; 54: 182-189.

Tahara H, Zitvogel L, Storkus WJ et al. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. J.Immunol. 1995; 154: 6466-6474.

Thanavala Y, Mahoney M, Pal S et al. Immunogenicity in humans of an edible vaccine for hepatitis B. Proc.Natl.Acad.Sci.U.S.A 2005; 102: 3378-3382.

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994; 84: 4008-4027.

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat.Rev.Immunol. 2003; 3: 133-146.

van Deurs B, Hansen SH, Petersen OW, Melby EL, Sandvig K. Endocytosis, intracellular transport and transcytosis of the toxic protein ricin by a polarized epithelium. Eur.J.Cell Biol. 1990; 51: 96-109.

van Herpen CM, Looman M, Zonneveld M et al. Intratumoral administration of recombinant human interleukin 12 in head and neck squamous cell carcinoma patients elicits a T-helper 1 profile in the locoregional lymph nodes. Clin.Cancer Res. 2004; 10: 2626-2635.

Villinger F. Cytokines as clinical adjuvants: how far are we? Expert.Rev.Vaccines. 2003; 2: 317-326.

Wagner B, Hufnagl K, Radauer C et al. Expression of the B subunit of the heat-labile enterotoxin of Escherichia coli in tobacco mosaic virus-infected Nicotiana benthamiana plants and its characterization as mucosal immunogen and adjuvant. J.Immunol.Methods 2004; 287: 203-215.

Wagner HJ, Bollard CM, Vigouroux S et al. A strategy for treatment of Epstein-Barr virus-positive Hodgkin's disease by targeting interleukin 12 to the tumor environment using tumor antigen-specific T cells. Cancer Gene Ther. 2004; 11: 81-91.

Walmsley AM, Alvarez ML, Jin Y et al. Expression of the B subunit of Escherichia coli heat-labile enterotoxin as a fusion protein in transgenic tomato. Plant Cell Rep. 2003; 21: 1020-1026.

Warzecha H, Mason HS, Lane C et al. Oral immunogenicity of human papillomavirus-like particles expressed in potato. J.Virol. 2003; 77: 8702-8711.

Webster DE, Cooney ML, Huang Z et al. Successful boosting of a DNA measles immunization with an oral plant-derived measles virus vaccine. J.Virol. 2002; 76: 7910-7912.

Wigdorovitz A, Carrillo C, Dus Santos MJ et al. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virology 1999; 255: 347-353.

Winter JA, Davies OR, Brown AP, Garnett MC, Stolnik S, Pritchard DI. The assessment of hookworm calreticulin as a potential vaccine for necatoriasis. Parasite Immunol. 2005; 27: 139-146.

Wolf SF, Temple PA, Kobayashi M et al. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J.Immunol. 1991; 146: 3074-3081.

Wong HL, Wilson DE, Jenson JC, Familletti PC, Stremlo DL, Gately MK. Characterization of a factor(s) which synergizes with recombinant interleukin 2 in promoting allogeneic human cytolytic T-lymphocyte responses *in vitro*. Cell Immunol. 1988; 111: 39-54.

Wong RJ, Chan MK, Yu Z et al. Effective intravenous therapy of murine pulmonary metastases with an oncolytic herpes virus expressing interleukin 12. Clin.Cancer Res. 2004; 10: 251-259.

Wu YZ, Li JT, Mou ZR et al. Oral immunization with rotavirus VP7 expressed in transgenic potatoes induced high titers of mucosal neutralizing IgA. Virology 2003; 313: 337-342.

Yoon C, Johnston SC, Tang J, Stahl M, Tobin JF, Somers WS. Charged residues dominate a unique interlocking topography in the heterodimeric cytokine interleukin-12. EMBO J. 2000; 19: 3530-3541.

Yuki Y, Kiyono H. New generation of mucosal adjuvants for the induction of protective immunity. Rev.Med.Virol. 2003; 13: 293-310.

Chapter Ⅱ

Expression of Bioactive Single-Chain Murine IL-12 in Transgenic plants

This chapter is presented in a format for submission to *Journal of Interferon and Cytokine Research*. Co-authors are Maureen C. Dolan [1], Michael J. Reidy [1,2] and Carole L. Cramer [1]

1. Arkansas Biosciences Institute, Arkansas State University, Jonesboro, AR72467
2. Department of Plant Pathology, Physiology and Weed Sciences,
Virginia Polytechnic Institute and State University, Blacksburg, VA24061
*Corresponding autho*r: ccramer@astate.edu

Ⅱ. 1 Abstract

Interleukin-12 (IL-12), an important immuno-modulator for cell-mediated immunity, shows significant potential as a vaccine adjuvant and anti-cancer therapeutic. However, its clinical application is limited in part by lack of an effective bioproduction system. Transgenic plants have been proved to be effective bioproduction systems for many biopharmaceuticals. Our research focused on expression of murine IL-12 in transgenic tobacco and examining its bioactivity *in vitro*. The functional IL-12 is a heterodimer consisting of two subunit, p35 and p40. To ensure stoichiometric expression of the two separately encoded, disulfide-linked subunits of IL-12 (p35 and p40), we expressed this cytokine in transgenic tobacco plants using a single-chain version of murine IL-12 (scmIL-12). From transgenic tobacco plants, hairy root cultures were developed and utilized for fast production of scmIL-12. The plant-derived scmIL-12 was characterized and purified for bioactivity assay *in vitro*. Our results demonstrated that the endogenous signal peptide of murine IL-12 has been cleaved off and it has been fully processed. Plant-derived scmIL-12 triggered induction of interferon-gamma (IFN-γ) secretion from mouse splenocytes as well as stimulation of splenocyte proliferation with comparable activities to those observed for the commercial mIL-12. Transgenic plants are able to produce bioactive scmIL-12 with abundant yield.

Ⅱ.2 Introduction

Interleukin-12 (IL-12), a potent pro-inflammatory cytokine critical for directing cell-mediated immunity, has significant potential as a prophylactic and immunotherapeutic adjuvant with clinical applications targeting both infectious disease and cancer. IL-12 plays a key role in polarizing the differentiation of T helper cells towards a T helper cell type 1 (Th1) immune response, directing or enhancing effector cells (CTL, NK, and macrophages) associated with cell-mediated immunity, and providing a critical link between innate resistance and adaptive immunity {Trinchieri, 1994 143 /id}. IL-12 induces T and NK cells to produce several cytokines and is particularly efficient at inducing IFN-γ production. Although IFN-γ is considered the signature cytokine of IL-12 activity, the attraction of IL-12 as an immune modulating therapeutic and vaccine adjuvant is its ability to direct cell-mediated immunity through its role in the activation, proliferation and memory of antigen-specific Th1 cells. As a vaccine adjuvant, IL-12 has been shown to enhance the efficacy of both systemic and mucosally delivered vaccines against human immunodeficiency virus, herpes simplex virus, feline immunodeficiency virus (FIV) and influenza including potential pandemic strains H5N1, H7N7 and H9N2 (Boyer et al., 2000; Leutenegger et al., 2000; Sin et al., 1999; Galarza et al., 2005). As an anti-

cancer therapeutic, IL-12 suppresses tumor growth, metastasis and angiogenesis (Noguchi et al., 1995; Zagozdzon et al., 1999; Gately and Brunda, 1995). In addition, IL-12's ability to mediate a shift away from Th2 (antibody) responses toward Th1 (cell mediated) responses may have applications in treatment of allergies and asthma (Gorbachev et al., 2001).

Although the pharmacokinetics of IL-12 are quite promising, clinical applications have been hindered by several factors including 1) dose- and schedule-dependent toxicity associated with the systemic administration of IL-12 (reviewed by Colombo et al., 2002) and 2) lack of a fully scalable production system for bioactive IL-12. Several strategies have emerged that successfully address the toxicity associated with systemic IL-12 administration by targeting "local" delivery via intranasal administration, gel matrix-mediated paracrine delivery, or DNA-based strategies (reviewed by Salem et al., 2006). However, large-scale bioproduction of fully functional IL-12 remains challenging, especially if one considers the potential scale and cost constraints that would be associated with widespread incorporation into vaccines targeting diseases such as pandemic influenza or AIDS. IL-12 is a complex heterodimeric glycoprotein that requires a eukaryotic production system to yield fully functional product. The ~70 kDa IL-12 heterodimer (p70) is comprised of two disulfide-linked subunits, p40 and p35, which are encoded by distinct genes (Gubler et al., 1991; Wolf et al., 1991). For murine IL-12 (mIL-12), the p40 subunit was shown to be a specific antagonist of the IL-12 heterodimer (Mattner et al., 1993). Single-chain IL-12 forms (p40-linker-p35), developed to address issues of stoichiometry and assembly in recombinant systems, retain IL-12 immuno-modulating and anti-tumor activities (Lieske et al., 1997; Anderson et al., 1997; Jiang et al., 1999; Foss et al., 1999) and have been utilized to produce recombinant IL-12 in yeast (Foss et al., 1999), mammalian cells (Lieschke et al., 1997; Anderson et al., 1997; Jiang et al., 1999) and plants (Gutierrez-Ortega et al., 2004).

We describe here the production of single chain mIL-12 (scmIL-12, Lieske et al., 1997) in transgenic tobacco plants and derived "hairy roots" and its purification and characterization including bioactivity assessments. Transgenic plants provide key scale, safety and cost advantages for production of IL-12 compared to animal cell-based production systems (reviewed in Cramer et al., 1999). Expression of both human and mouse single chain IL-12 in plant cells or transgenic plants has been reported previously (Gutierrez-Ortega et al., 2004, 2005; Kwon et al., 2003). However, IL-12 protein yields described in these reports were so low that they did not support purification or specific activity comparisons with animal-derived IL-12. We have developed IL-12 expression lines at levels ~500 fold higher allowing extensive characterization of the plant-derived product and its immunomodulating

activity. Our results suggest that plant-derived mIL-12 has equivalent activity to that of commercially available animal cell-derived heterodimeric mIL-12 based on cell proliferation assays and the induction of interferon-γ in mouse splenocytes.

Ⅱ.3 Material and Methods

Ⅱ.3.1 Construction of the IL-12 gene

Plasmid pSFG-mIL-12.p40.L.delta.p35 (Lieschke et al., 1997; kindly provided by Dr. Mulligan) was the source for single chain murine IL-12 (scmIL-12). Flanking restriction enzyme sites, *Kpn*I and *Sac*I, were added to the scmIL-12 sequence by PCR with primers 5'-GGTACCATGGGTCCTCAGAAGCTAA-3' and 5'-GAGCTCTCAGGCGGAACTCAGATAG-3'. Following sequence confirmation, this 1.6 kb *Kpn*I / *Sac*I fragment was inserted into a cloning vector pBC (Stratagene) previously modified to contain the constitutive plant promoter (double enhanced 35S promoter; Lam et al., 1989) and a translational enhancer (TEV, Carrington et al., 1990); the resulting construct was designated *scmIL-12* (Fig.Ⅱ. 1 A). The plant expression cassette was then excised with *Hin*dⅢ and *Sst*I and cloned into a plant binary vector pBIB-Kan (Becker, 1990) for transformation and expression in plants.

Ⅱ.3.2 Production of scmIL-12 transformed plants and hairy roots

The *scmIL-12* expression vector was mobilized into the *Agrobacterium tumefaciens* strain LBA4404 by a modified freeze-thaw method (Chen et al., 1994). Transformation of *Nicotiana tabacum* cv. *Xanthi* was performed using a petiole transformation procedure as previously described (Medina-Bolivar and Cramer, 2004). Following a 48 hour incubation of *Agrobacterium*-infected leaf explants on Murashige and Skoog Basal Salt media (MS; GIBCO), leaves were transferred to supplemented MS media (0.1 mg/l 1-naphthalene acetic acid, 1mg/l 6-benzylamine purine, 500 mg/l carbenicillin and 250 mg/l kanamycin) to facilitate transgene integration into plant cells and to provide selection of regenerated transgenic shoots. Antibiotic-selected plantlets were screened for the presence of transgene by PCR using m*IL-12* specific primers (as described above). Of the 66 independent transgenic plants generated, those with highest mIL-12 expression levels (see ELISA screening below) were maintained for sterile propagation as fully rooted plants in sterile agar media. Select lines were also transferred to soil and taken to seed. Analogous cultures of non-transgenic tobacco plants were maintained and used as controls for this study.

Hairy roots from select, high-expressing transgenic plants (I8-2, I8-4 and I8-9) and wild type plant were also established by a procedure previously described (Medina-Bolivar and Cramer, 2004). Briefly, *Agrobacterium rhizogenes* (ATCC 15834) was introduced at wound sites created by cutting longitudinally along the midrib of excised scmIL-12 expressing transgenic leaves. Infected leaves were incubated on solid MS media for two weeks to allow hairy root development at wound site. Individual root tips representing independent hairy root clonal lines were excised and transferred to B5 medium containing cefotaxime to remove *Agrobacterium rhizogenes*. Liquid cultures were initiated with ~20 root tips (1cm) in a 250 ml flask containing B5 media (50ml) and maintained under continuous light with shaking (90 rpm). For routine, scale-up production of mIL-12, 1-week old hairy root cultures (50 ml media/250 ml flask) were transferred to PYREX® 2800 mL Fernbach-Style Culture Flask (Item #4420-2XL, Corning) containing 0.5 L media and cultured for an additional 2 weeks. Typically each flask yielded 20-30 g of root biomass; tissue was stored at -80 ˚C upon harvest.

Ⅱ.3.3 IL-12 ELISA

A heterodimer-specific mIL-12 enzyme-linked immunoabsorbant assay (ELISA) was used for the detection and quantitation of plant-derived mIL-12 (R&D Systems). For purposes of this paper, this assay will be referred to as the "conformational" mIL-12p70 ELISA for it exclusively detects only correctly folded and/or assembled mIL-12 p70 protein. Plant tissue was ground in 2 volumes of extraction buffer (100 mM Tris base, 100 mM ascorbic acid, 150 mM NaCl, 4 mM EDTA, 2.5% PVP-40 0.1% Tween 20, pH7.0) and cell-free supernatants were analyzed on Immunlon 4 HBX plates (Thermo Labsystems) coated with 1 μg/ml rat anti-mIL12 p70 monoclonal capture antibody (clone 48110.111; R&D Systems). Following a block step in PBST (0.1% of Tween 20 in PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) with 1% BSA, serial dilutions of standard recombinant mIL-12 (R&D Systems), plant-derived mIL-12 samples and equivalent extracts from non-transgenic roots were loaded. Captured mIL-12 p70 was detected with 100 ng/ml biotinylated anti-mIL-12 antibody (R&D Systems), streptavidin HRP (R&D Systems), and KPL substrate solution. Plates were read at 450nm. All quantitation, purification screens and specific activity calculations for plant-derived mIL-12 were based on this conformational p70 ELISA using the commercially-available insect cell-derived mIL-12 as standard (R&D Systems).

Ⅱ.3.4 SDS-PAGE and western blot analysis

Plant tissue was ground in extraction buffer (same as above for mIL-12 ELISA), total protein determined (Advanced protein assay reagent, Cytoskeleton Inc.) and 5 μg of total soluble

protein/sample were resolved on a 10% SDS-polyacrylamide gel (Invitrogen), with or without reducing agent (100mM DTT), in 1×SDS gel loading buffer. Protein gels were either silver stained (FAST*silver*, GEBiosciences) or blotted onto nitrocellulose membranes (Bio-Rad) for western analyses. For immunoblot detection of the p70 heterodimer and the p40 and p35 subunits, the primary antibody used was a goat anti-mouse IL-12 antibody (R&D Systems) at 1:10,000 and alkaline phosphatase-conjugated rabbit anti-goat IgG (Bio-Rad) served as the secondary detection antibody. For western blot analysis that exclusively detected the heterodimer, a rat anti-mIL-12 p70 (clone#48110.111) primary antibody at 1:1000 and an alkaline phosphatase-conjugated rabbit anti-rat IgG secondary antibody at 1:1000 were used. Detection on immunoblots was carried out using the CDP-Star (Roche) and Nitroblock Enhancer Ⅱ (TROPIX, Bedford, MA) system in accordance with manufacturers' procedures. Commercially available insect cell-derived mIL-12 (R&D Systems) was utilized as the positive control for all mIL-12 western blot analyses while carrier-free CHO-cell derived protein (PeproTech) served as the standard on silver stained analyses.

Ⅱ.3.5 Purification of plant-derived mIL-12

Hairy root tissue was ground in liquid nitrogen and homogenized with 2 volumes of grinding buffer (100 mM Tris base, 100 mM ascorbic acid, 150 mM NaCl, 4 mM EDTA, 2.5% PVP-40, 0.1% Tween 20, 100 uM PMSF, pH 7.6). After centrifugation, supernatants were precipitated by 20% polyethylene glycol (PEG-8000). PEG precipitate was resuspended in phosphate buffer (50mM phosphate buffer, pH 7.6) and filtered through a 0.45 μm membrane before application onto a cation exchange column (Unosphere S; Bio-Rad). Plant-derived mIL-12 was eluted with a 0-1 M NaCl salt gradient in phosphate buffer (pH 7.6). Fractions containing mIL-12 were determined using the p70 conformational ELISA, pooled, dialyzed against 50 mM Tris-Cl (pH 7.6) and applied to an anion exchange column (Uno Q1; Bio-Rad). Following elution with a 0-1M NaCl gradient in Tris-Cl (pH 7.6) buffer, pooled fractions containing mIL-12 were concentrated (Centricon YM-30, Millipore) and further purified by gel filtration on a SEC125 column (Bio-Rad). Fractions containing mIL-12 were quantified by mIL-12 p70 ELISA (R&D Systems), lyophilized to dryness (LabConco) and stored at 4˚C. Qualitative and purity assessments of plant-derived mIL-12 were evaluated in comparison to CHO cell-derived mIL-12 (PeproTech) by non-reducing SDS-PAGE and visualized by silver stain.

Ⅱ.3.6 N-terminal sequencing

N-terminal sequencing of plant-derived mIL-12 was performed by the Biomolecular Research Facility at University of Virginia (Charlottesville, VA). Purified plant-derived mIL-12 for analysis

was subjected to SDS-PAGE and transblotted onto Sequi-Blot PVDF membrane (Bio-Rad). Membranes were stained with Coomassie Brilliant Blue and bands of interest were excised for Edman chemistry-based sequencing.

Ⅱ.3.7 Endoglycosidase digestion

To establish the presence of asparagine-linked glycans, 1 μg of purified plant-derived mIL-12 protein was subject to a denaturing process and subsequently digested with 250U of endoglycosidase (PNGase F or Endo H) at 37 ˚C for 1 hour as described in manufacturer's protocol (New England Biolabs). Digests were analyzed by SDS-PAGE under reducing conditions and compared to digests of animal cell-derived mIL-12 (Peprotech, Inc.).

Ⅱ.3.8 Mouse IFN-γ secretion assay

All mice were housed in Arkansas Biosciences Institute Animal Facility at Arkansas State University. The protocol was approved by Arkansas State University Animal Care Committee. Unfractionated mouse splenocytes isolated and pooled from two to three 8-20 weeks old female Balb/c mice (Jackson Laboratory) were cultured in complete RPMI 1640 (5 mM HEPES, 2 mM glutamine and 10% heat inactivated FBS and penicillin/streptomycin; Gibco) supplemented with 10 ng/ml rhIL-2 (R&D Systems) at 37 °C /5% CO_2. Splenocytes were plated at 5×10^5 cells/well in 96-well plates and incubated for 48 hours with varying amounts of purified plant-derived scmIL-12, corresponding amounts of commercial animal-cell derived mIL-12 (R&D Systems) or equivalent fractions derived from non-transgenic root cultures. Samples were run in triplicate; mIL-12 amounts used were based on the conformational p70 ELISA. For quantitative comparison of IFN-γ production and secretion, supernatants were collected and tested by IFN-γ ELISA according to manufacture's instructions (R&D Systems). The results are given as mean±1 standard deviation.

This assay was repeated numerous times using two independent sources of animal cell-derived mIL-12 standard (CHO-cell and insect cell-derived). In addition IFN-γ secretion in response to plant-derived mIL-12 was assessed for splenocytes established from both inbred strains (Balb/c, C57BL/6) and an outbred strain (ICR) of mice purchased from Jackson Laboratory and Harlan Laboratory.

Ⅱ.3.9 Splenocyte proliferation assay

The proliferation response of mouse splenocytes to plant-derived mIL-12 was measured for equivalent bioactivity to the animal cell-derived mIL-12 standard (R&D Systems). Briefly, mouse splenocytes were isolated and plated at a concentration of 7.5×10^5/ml in media (RPMI 1640, 5 mM

HEPES, 2 mM glutamine, 10% heat inactivated FBS, penicillin/streptomycin, 50 μM 2-ME, 10 ng/ml rhIL-2) and cultured for 3 days in the presence of the mitogen, PHA (10 μg/ml; Sigma). Splenocytes were collected, washed and resuspended in assay media (RPMI 1640, 20 ng/ml PMA, 5 mM HEPES, 2 mM glutamine, 10% heat inactivated FBS, penicillin/streptomycin and 50 μM 2-ME) and plated at 100 μl of 4×10^5 cells/ml with various concentrations of animal cell-derived mIL-12 (R&D systems) or equivalent amounts of plant-derived samples (quantitation based on mIL-12 ELISA detailed above). Following a 2-day culture period, cell proliferation rates were compared in a standard colorimetric assay analyzed at O.D. 490 nm (Promega Substrate CellTiter 96 Aqueous One Solution Reagent). The results are given as mean±1 standard deviation.

Ⅱ.4 Results

Ⅱ.4.1 Production of mIL-12 in transgenic plants

We utilized the well described functional single chain form of mouse IL-12 (scmIL-12; kindly provided by Dr. Richard Mulligan, Harvard Medical School) for expression in plant in order to facilitate stoichiometric subunit assembly. This construct links the p40 and mature p35 subunit genes with a $(Gly_4Ser)_3$-encoding polylinker (Lieske et al., 1997). The scmIL-12 sequence was PCR-amplified in order to provided flanking *Kpn*I and *Sst*I sites and cloned downstream of the strong constitutive *de35S* promoter [the dual-enhanced 35S promoter] (Lam et al., 1989) with the tobacco etch virus (*TEV*) translational enhancer (Carrington et al., 1990). Following DNA sequence confirmation, the *Hin*dⅢ / *Sst*I cassette containing the promoter:scmIL-12 sequence (Fig. Ⅱ.1 A) was subsequently ligated into pBIB-Kan (Becker, 1990), a plant transformation vector that provided plant terminator sequences, an adjacent NPT Ⅱ selectable marker, and flanking T-DNA border sequences that delineate the region transferred into the plant cell by *Agrobacterium tumefaciens*-mediated transformation. This vector was mobilized into *A. tumefaciens* strain LBA4404 and used to transform tobacco leaf material (Medina-Bolivar et al., 2004). Because transgene expression levels can vary depending on site of insertion, we generated more than 60 independent transgenic lines to ensure identification of high expressers.

Leaf tissue from presumptive transgenic plant lines was initially screened for the presence of the transgene by PCR using *mIL-12* specific primers (data not shown) and subsequently analyzed using an anti-mouse IL-12 p70 ELISA (Fig. Ⅱ.1 B) in order to identify high expressing lines. In addition to providing an effective quantitation tool for mIL-12 protein levels, this ELISA also provided

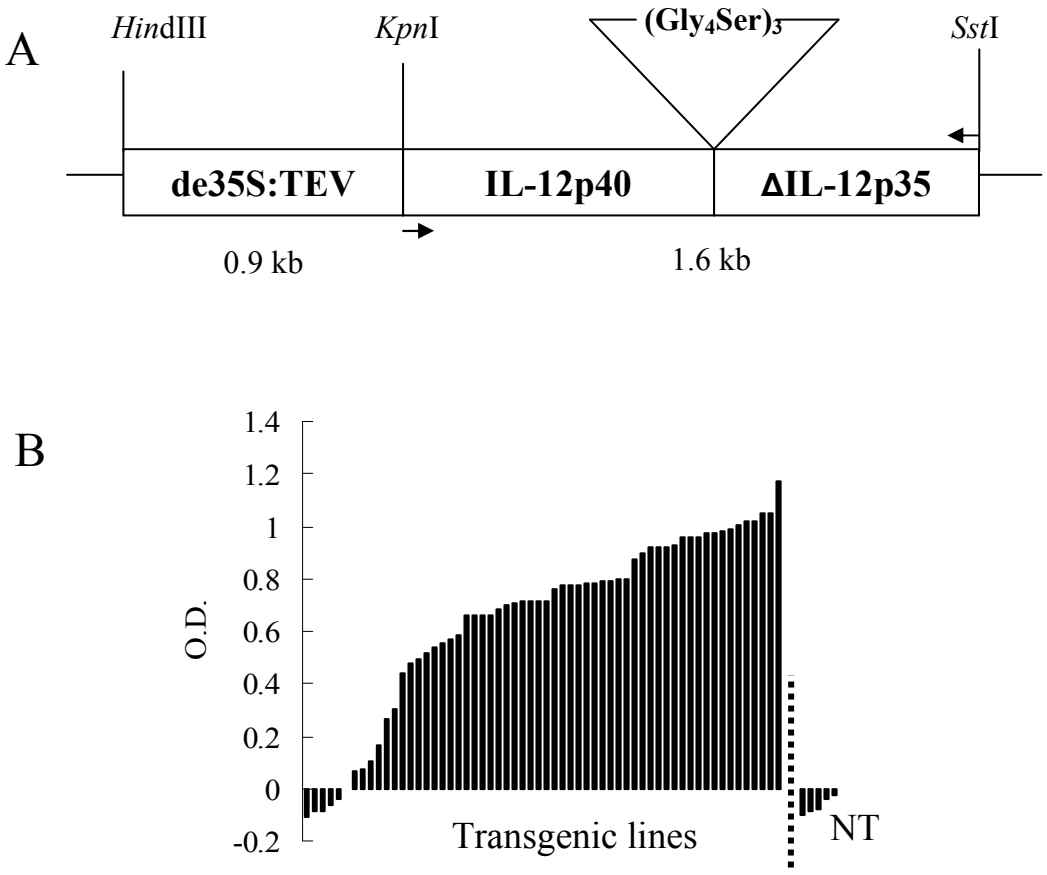


Fig. Ⅱ.1 Generation and screening of transgenic plants expressing murine IL-12. (A) Construct of *scmIL-12* that was utilized to transform tobacco plants through *Agrobacterium*-mediated transformation. *de35S:TEV*: double enhanced 35S promoter with TEV translational enhancer; *IL-12p40*: DNA fragment encoding p40 subunit with native mouse signal peptide; *ΔIL-12p35*: DNA fragment encoding the mature p35 subunit (without signal peptide). (B) Relative IL-12 levels in leaf extracts of independent transgenic plant lines. IL-12 levels were determined in crude leaf extracts by IL-12 ELISA. Five bars on the right side of the dot line represent readings from non-transgenic (NT) control plants.

conformational data since this mIL-12 p70 antibody only recognizes correctly folded mIL-12 heterodimer (Manufacturer's instruction). Of the 66 regenerated plants, 55 contained detectable levels of mIL-12 (Fig.Ⅱ.1 B). The highest expressing lines contained mIL-12 at levels of 3-5μg/mg of total soluble proteins (25-40μg/g leaf fresh weight) and contained 1-2 copies of the IL-12 transgene as determined by genomic Southern analyses (data not shown). Selected high-expressing plant lines were transferred to soil for seed production.

Several high expressing mIL-12 plants were used to establish hairy root cultures. We commonly use this self-replicating root system for initial product characterization due to its utility in providing rapid biomass accumulation, developmental consistency yielding very predictable protein recovery, and "feedstock" characteristics that facilitate protein purification (Medina-Bolivar and Cramer, 2004). Yields of full-length p70 mIL-12 product in crude extracts from hairy root cultures was higher (~16 μg/mg total soluble protein; 33 μg/g fresh weight) than that derived from leaf material of the same line based on the "conformational" mIL-12 ELISA.

Ⅱ.4.2 Characterization of plant-derived mIL-12 product

Initial characterization of mIL-12 in crude extracts of both leaf tissue and cultured hairy roots was performed using western immunoblot analysis. As shown in Fig.Ⅱ.2 A, anti-mIL-12 neutralizing polyclonal antibody (R&D Systems), which cross-reacts with both intact mIL-12 heterodimer (p70) and the individual subunits (p40 and p35), detected the presence of a strongly cross-reacting product of approximately 70 kDa in crude extracts of mIL-12-expressing plants that was absent in protein extracts from non-transgenic control plants. The mobility of plant-derived scmIL-12 (pdIL-12) was similar to that of commercial recombinant mIL-12 p70 (R&D Systems, insect cell-derived heterodimer) with a slight shift in size consistent with the presence of the linker sequences and possible variation in glycan composition. In addition to the dominant full length mIL-12 p70 product, a minor band migrating at ~40 kDa also cross reacted with mIL-12 antibody and is likely a breakdown of the single chain pdIL-12 (Fig.Ⅱ.2 A). As shown in Fig.Ⅱ.2 B, the pdIL-12 70 kDa band present in these non-reducing gels also cross-reacted with the "conformational" anti-p70 monoclonal antibody specific for the assembled form of mIL-12. Analogous p70 mIL-12 product was detected from extracts derived from hairy root cultures (data not shown).

To enable a truly comparative demonstration of plant-derived mIL-12 bioactivity to that of commercially available animal-cell derived IL-12, purification of plant-derived mIL-12 was required. Hairy root cultures were selected as the pdIL-12 source due to higher levels and ease of producing

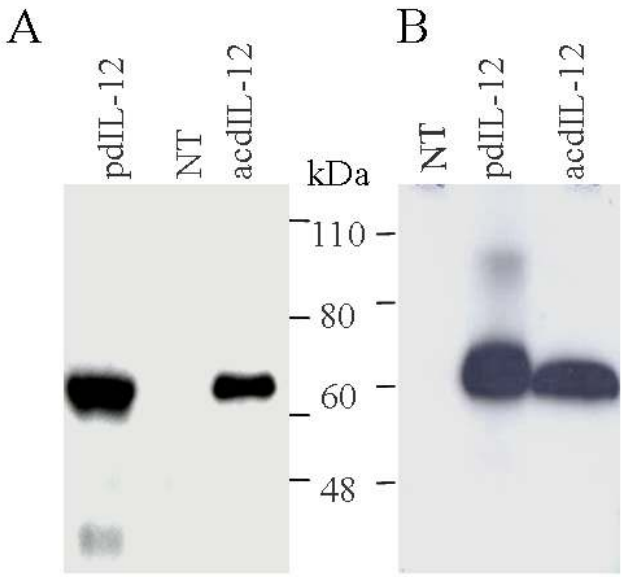
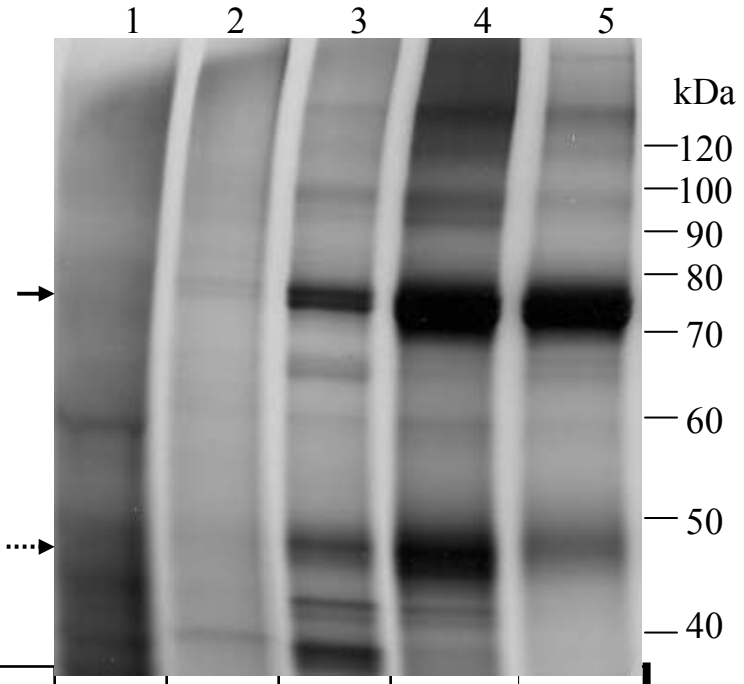


Fig. Ⅱ.2 Western blotting analysis of IL-12 from transgenic plants. (A) Proteins from crude extract (20 μg) of IL-12-expressing plants (pdIL-12), from non-transgenic control plants (NT), and animal cell-derived IL-12 (acdIL-12, 50 ng) were resolved on a 10% SDS-PAGE gel under non-reducing conditions and transferred to nitrocellulose membrane. The membrane was probed with polyclonal mIL-12 antibody (A), or a monoclonal antibody (anti-mIL-12 p70) specific for conformationally intact p70 (B).

consistent biomass. Our initial purification efforts focused on optimizing extraction buffers that addressed issues of pH, ionic strength, and stabilizing components (i.e. protease inhibitors, PVP) to maximize protein recovery yet maintaining compatible conditions for downstream purification processes. Plant-derived scmIL-12 exhibited similar binding properties and elution profiles on anion exchange and cation exchange chromatography to that of IL-12 expressed in other systems (Kobayashi et al., 1989; Foss et al., 1999; Manner et al., 1994). Fig.Ⅱ.3 highlights representative samples resolved by silver-stained SDS-PAGE for each step of the purification scheme of plant-derived scmIL-12 with corresponding recovery and purification. This purification regime typically yields about 4μg of recombinant mIL-12 per g roots fresh weight (150 μg purified mIL-12 per 1 liter culture), representing a 150 fold-enrichment and a purity exceeding 90%. For long-term storage, purified plant-derived scmIL-12 can be lyophilized without obvious loss in biological activity (data not shown). This purification scheme has demonstrated similar performance and recovery rates for mIL-12 from transgenic plant leaf tissue (data not shown).

Murine IL-12 is a complex glycoprotein that is targeted through the endomembrane system, glycosylated, and secreted in its native system. Murine IL-12 p70 was detected in the media of mIL-12-expressing hairy root cultures (data not shown) suggesting that it is targeted for secretion in plants. However, only 1-3% of the total mIL-12 produced was recovered from the media of late log hairy root cultures suggesting that this large protein may be inefficiently released from plant cell walls and/or it may be susceptible to proteolytic breakdown in the media. To further address whether plants effectively target mIL-12 to the endomembrane system and correctly process the mouse signal peptide, we performed amino-terminal sequence analyses on purified plant-derived mIL-12 resolved on a reducing SDS-PAGE gel. Under reducing conditions, a significant proportion of the p70 product migrates at lower molecular sizes consistent with potential cleavage within the linker region between p40 and p35. The N-terminal sequence generated from the “p40” band was MWELEK…, corresponding with the expected mature p40 subunit. This result demonstrated that the endogenous mouse p40 signal peptide was effectively cleaved in plant cells and confirmed that the purified product is in fact mIL-12. A second N-terminal sequence, SGGGGSG, was recovered from a lower molecular size band and suggests cleavage occurred in the linker positioned upstream of the p35 subunit.

Functional IL-12 has only been successfully expressed in eukaryotic systems, suggesting that posttranslational modification (likely glycosylation) of IL-12 is critical to its functional expression. In order to confirm that mIL-12 undergoes N-linked glycosylation in tobacco cells, we analyzed apparent molecular size profiles of plant- and animal cell-derived mIL-12 before and after treatment with the



Specific activity (μg/mg)	4.47	14	133	642	690	**Overall recovery and purification**
Recovery (%)	100	82	41	53	45	**8**
Enrichment	-	3.12	9.55	4.81	1.07	**154**

Fig. Ⅱ.3 Purification of IL-12 from hairy root tissue. Proteins present in crude root extracts and various concentrated purification fractions were resolved on a 10% SDS-PAGE gel under non-reducing conditions and visualized by silver staining. The black arrow shows the expected location of IL-12 p70 and the black dash arrow points to IL-12 p40. Lanes include crude extracts (1) and IL-12 containing fractions following polyethylene glycol precipitation (2), S cation exchange chromatography (3), Q anion exchange chromatography (4), and size-exclusion column chromatography (5). Data below each lane indicates IL-12 specific activity, recovery and enrichment at each step for a representative purification.

glycan-degrading enzymes, endoglycosidase H (EndoH) and peptide-N-glycosidase F (PNGaseF). Size differences in enzymatically digested products were not effectively resolved for the p70 product; therefore we focused on the p40 subunit profile resolved under denaturing conditions. A reduction in molecular size of IL-12 p40 following treatment with EndoH or PNGaseF confirmed the presence of N-linked glycans on the plant-derived recombinant cytokine (Fig.Ⅱ.4). The EndoH pattern for the plant-derived and animal cell-derived mIL-12 was similar suggesting the presence of high-mannose or hybrid forms on mIL-12 synthesized in both species. Whereas PNGaseF digested the animal cell-derived mIL-12 to a single p40 product, two bands were evident in the plant-derived sample (Fig.Ⅱ.4). The higher molecular weight band may be attributed to possible variations of cleavage within the glycine-serine linker or to the presence of α-1,3 fucose, a sugar present on some complex plant N-linked glycans that is known to be inefficiently cleaved by PNGaseF (Tretter et al., 1991).

Ⅱ.4.3 Bioactivity of plant-derived mIL-12

Paramount to demonstrating the utility of plants in the bioproduction of complex mammalian proteins such as mIL-12, is establishing bioequivalent function of the purified plant-derived cytokine with a characterized commercially-available mIL-12 (Fig.Ⅱ.5 A). Among the important roles of this cytokine, IL-12 acts on T and NK cells in stimulating proliferation and inducing production of interferon-gamma (IFN-γ), activities that are used as signature assays of IL-12 bioactivity. Secretion of IFN-γ was induced in pre-activated mouse splenocytes treated with purified plant-derived mIL-12 and the response kinetics were similar to that elicited by animal cell-derived mIL12 (Fig.Ⅱ.5 B). Equivalent fractions derived from non-transgenic plant extracts did not induce IFN-γ secretion. In order to demonstrate that the increased secretion of IFN- γ was in fact attributed to plant-derived mIL-12, neutralizing mIL-12 antibodies were incorporated into these assays and shown to completely block the activity of pdIL-12 (Fig.Ⅱ.C). Plant-derived mIL-12 also supported proliferation of pre-activated splenocytes in a dose-dependent manner that mirrored the response of commercially available recombinant mIL-12 (acdIL-12, R&D Systems) and was effectively blocked with neutralizing mIL-12 antibodies (Fig.Ⅱ.6). Moreover, plant-derived mIL-12, showed similar biological activity among different inbred (Balb/c, C57BL/6) and outbred (ICR) mouse strains that mirrored secretion profiles induced with animal-derived mIL-12 (Fig.Ⅱ. 7).

In conclusion, we have identified tobacco lines that express the mouse single chain IL-12 at high levels and have purified the mIL-12 product to sufficient levels to enable bioactivity assessments

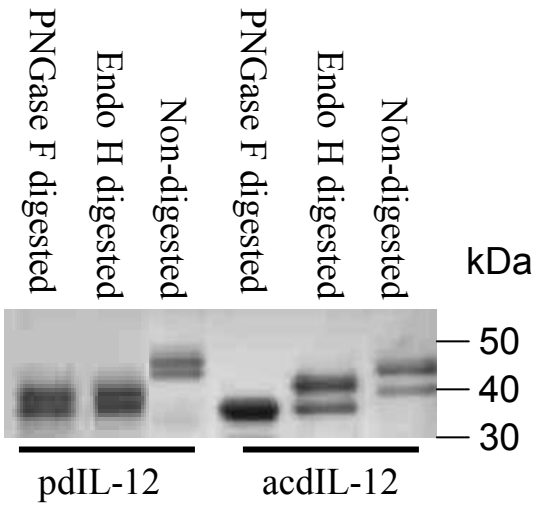


Fig. Ⅱ.4 Endoglycosidase digestion of plant-derived mIL-12 and animal cell-derived mIL-12 (acdIL-12). About 1 μg of purified plant-derived mIL-12 (pdIL-12), or animal cell-derived (acdIL-12), was digested by endoglycosidase (PNGase F or Endo H) at 37 ºC for one hour. The endoglycosidase-digested proteins and non-digested mIL-12 (pdIL-12 and acdIL-12) were resolved on a 12% SDS-PAGE gel under reducing condition and visualized by silver stain.

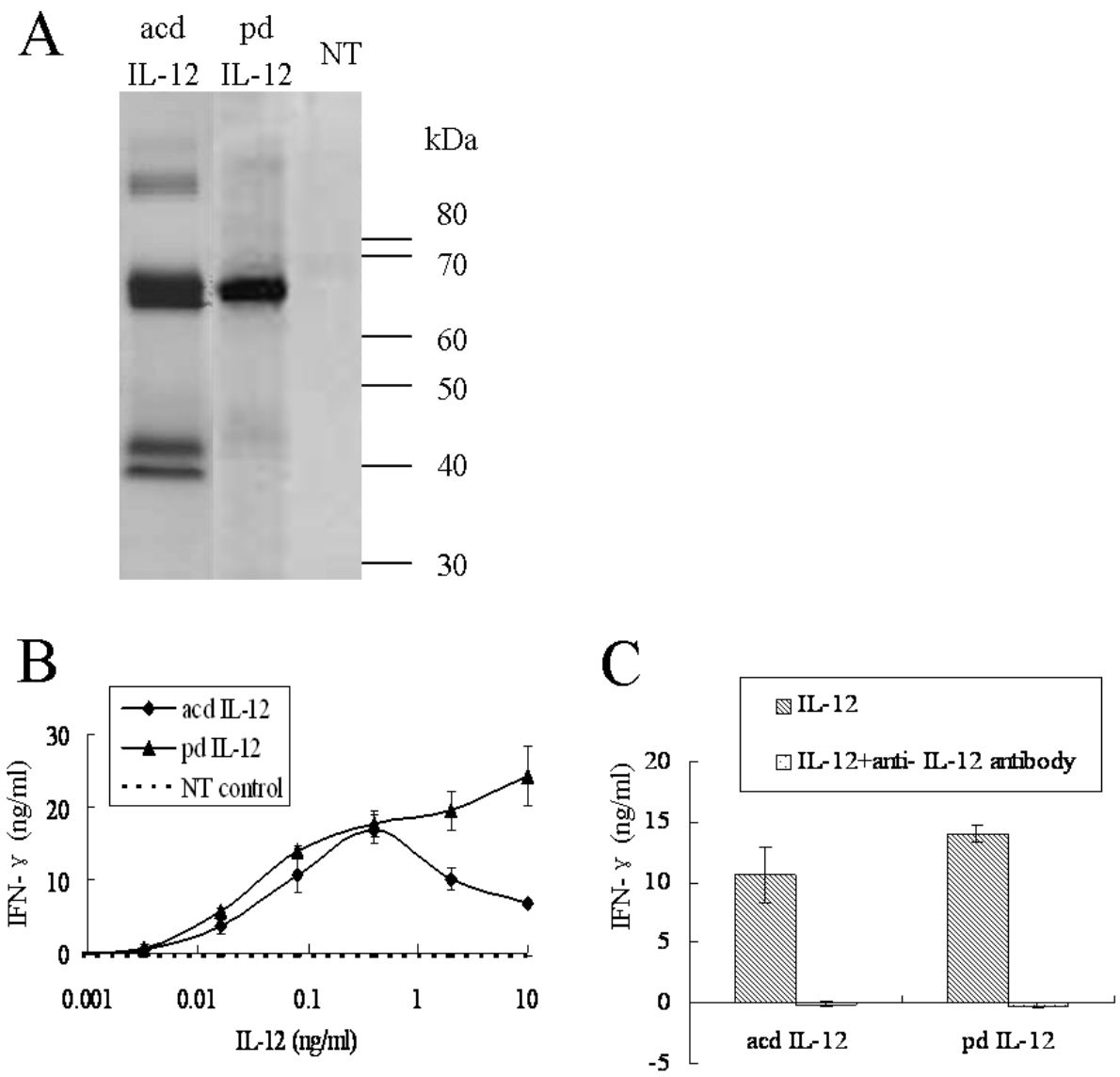


Fig. Ⅱ.5 IL-12-mediated induction of interferon-gamma (IFN- γ) in mouse splenocytes. (A) Comparison of plant-derived mIL-12 to animal cell-derived mIL-12 by SDS-PAGE. About 1 μg of animal-cell-derived mIL-12 (acdIL-12), plant-derived mIL-12 (pdIL-12) and equivalent fractions from non-transgenic control (NT) were loaded onto 10% SDS-PAGE gel under non-reducing condition, and visualized by silver stain. (B) Induction of IFN- γ in mouse splenocytes in response to mIL-12. Splenocytes from Balb/c mice were cultured for 48 hours, with 10 ng/ml rhIL-2 and the indicated amounts of animal cell-derived mIL-12 (acdIL-12), purified mIL-12 from transgenic hairy roots (pdIL-12), or equivalent fractions from non-transgenic control (NT control). Supernatants were assayed for IFN- γ concentration by ELISA. (C) Induction of IFN-γ in the presence or absence of IL-12 neutralizing antibody. Murine IL-12 from different sources was incubated with or without mIL-12 neutralizing antibody for 2 hours prior to addition to mouse splenocyte cultures. The procedure of cell culture was the same as above. Data from 0.08 ng/ml of IL-12 is shown. Data points represent the mean ±1 standard deviation of triplicate wells. These data are representative of three separate experiments.

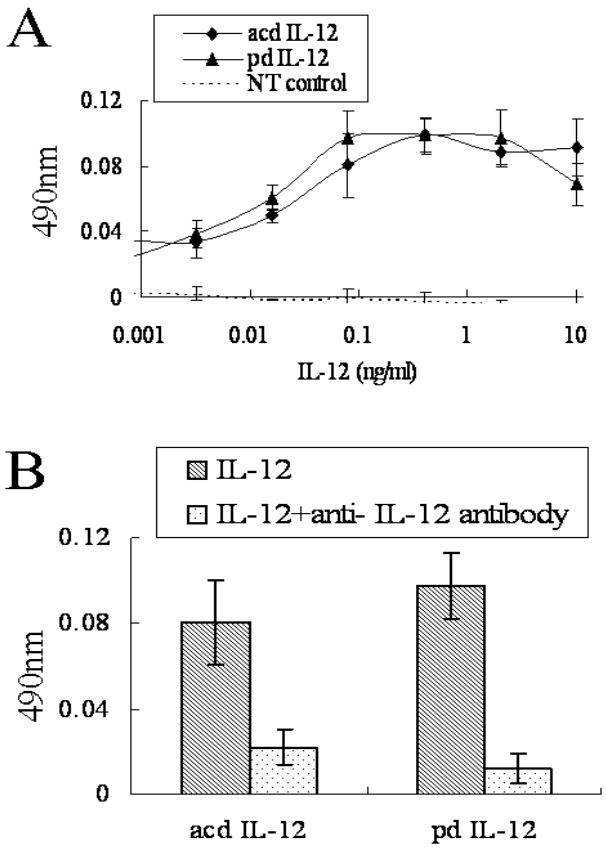


Fig. Ⅱ.6 IL-12-stimulated proliferation of mouse splenocytes. (A) Standard colorimetric cell proliferation assays were performed on splenocytes from Balb/c mice as described in Material and Methods. PHA-preactivated splenocytes were cultured for 48 hours with 10 ng/ml rhIL-2 and the indicated amounts of animal cell derived mIL-12 (acdIL-12), purified mIL-12 from transgenic hairy roots (pdIL-12), or equivalent fractions from non-transgenic control (NT control). (B) Comparison of cell proliferation in splenocytes treated with mIL-12 that was pre-incubated with or without neutralizing antibodies. The procedure of cell culture and cell proliferation assays was the same as above. Data from 0.08 ng/ml of IL-12 is shown. Data points represent the mean ±1 standard deviation of triplicate wells. These data are representative of three separate experiments.

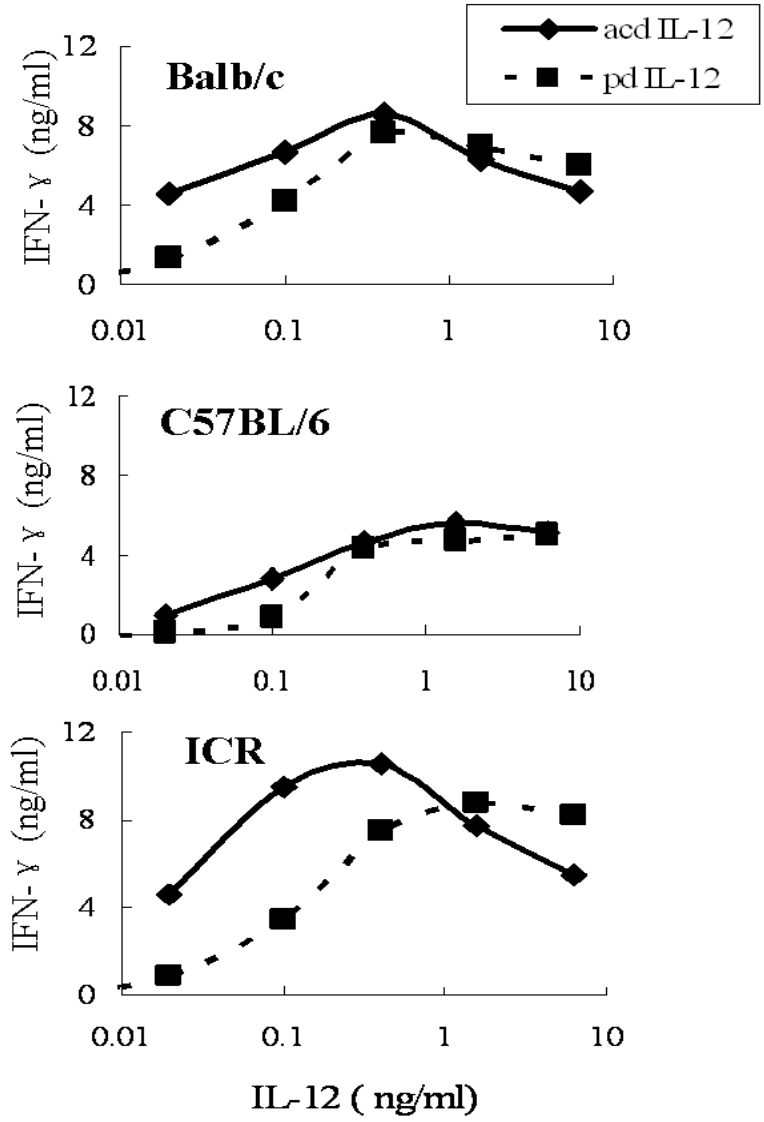


Fig. Ⅱ.7 Plant-derived mIL-12 and animal-cell-derived mIL-12 show similar kinetics of IFN- γ induction in splenocytes derived from multiple mouse strains. Splenocytes pooled from two to three Balb/c, C57BL/6, or ICR mice, were cultured for 48 hours, with 10 ng/ml rhIL-2 and the indicated amounts of animal cell derived mIL-12 (acdIL-12), purified mIL-12 from transgenic hairy roots (pdIL-12). Supernatants were assayed for IFN- γ concentration by ELISA.

and direct comparisons with mIL-12 derived from animal (CHO or insect) cell cultures. Our data on biological reactivity using the signature *in vitro* IL-12 readouts of IFN-γ induction and secretion and cell proliferation demonstrate analogous bioactivity and response kinetics between plant- and animal cell-derived mIL-12.

Ⅱ.5 Discussion

IL-12 is a very important immunomodulator with significant potential in clinical application. The recombinant expression of IL-12 in different expression systems has been investigated (Foss et al., 1999; Lieschke et al., 1997; Gutierrez-Ortega et al., 2004; 2005; Kwon et al., 2003; Bermudez-Humaran et al., 2003). Our work provides a cost-effective and scalable production system for IL-12. We utilized a murine single chain IL-12 gene in our plant production system due to the consideration of simple transformation, stoichiometric subunit assembly and bioactivity observation in mouse model. This research has not only demonstrated the capacity of a plant bioproduction platform to produce large amounts of purified murine IL-12 (pdIL-12), but has also shown that pdIL-12 exhibits full bioactivity. We developed a purification scheme for pdIL-12 which enabled accurate assessment of cytokine bioactivity. By studying the activity of purified pdIL-12 in mouse splenocytes culture, we proved that pdIL-12 induced mouse splenic cells to secrete IFN-gamma and stimulated the proliferation of splenocytes, exhibiting equal *in vitro* bioactivity to mammalian cell-derived (data not shown) and insect cell-derived mIL-12. Purification of pdIL-12 is critical to its bioactivity assay, especially in the splenocytes proliferation assay because wild-type plant crude extracts also stimulate the proliferation of splenocytes (Gutierrez-Ortega et al., 2004).

Previous investigations have produced IL-12 in transgenic plants (Gutierrez-Ortega et al., 2004; 2005; Kwon et al., 2003). However, their expression levels were 10-100 folds lower than what we observed in our work. After comparing our research to that of those two research groups, we believe that a number of factors may have contributed to these differences in expression and recovery of IL-12 including differences in IL-12 gene source (mouse rather than human), transformation methods, numbers of plants screened, and selection/optimization of extraction buffers. We utilized a murine single chain IL-12 gene in our research and transformed tobacco plants with a modified *Agrobacterium*-mediated transformation. After screening 66 transformants, we identified the high expresser which produce ~30 μg of IL-12 per gram of fresh tissue. Moreover, the extraction buffer we used has much higher buffer capacity than that used by Gutierrez-Ortega et al. (2004) in their work, which probably assisted the release of IL-12 from cell walls.

IL-12 is a complex protein that requires post-translational modifications for its activity. We have shown that IL-12 synthesized in transgenic plant is indeed functional and that plants perform the necessary folding and post-translational processing required for activity. Murine IL-12 produced in transgenic plants appeared to be a cluster of similar sized molecules at around 70kD (primarily two bands), probably due to different glycoforms. This phenomenon has also been observed in other IL-12 production systems (Foss et al., 1999; Lieschke et al., 1997). N-terminal sequencing of plant-derived scmIL-12 confirmed the identity of the protein and revealed that the mouse p40 peptide was correctly cleaved in plants. The existence of mIL-12 in hairy root media also confirmed that the endogenous signal-peptide of mIL-12 is functional in plants and is capable of directing IL-12 into the endomembrane system that leads to secretion of IL-12 into the apoplast (data not shown). Foss et al. (1999) has shown that *Pichia pastoris* can produce bioactive porcine IL-12 but it retains the signal peptide. Our results suggest that plants can effectively recognize and process mammalian signal peptides.

Hairy roots have been investigated as model culture systems for natural products, small molecules and proteins (Shanks and Morgan, 1999). This research also shows that hairy roots are ideal production systems for lab-scale recombinant protein expression. We utilized hairy roots as the production system for two reasons. Firstly, hairy roots rapidly accumulate genetically identical biomass. This permits characterization of plant-derived mIL-12 from hairy roots while the transgenic plants are growing to seeds in the greenhouse. Secondly, hairy roots do not express ibulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the major protein of photosynthesis which comprises about 50% of total leaf proteins in tobacco. The resulting simplified protein background of hairy roots facilitates the purification of recombinant proteins.

Plants have been investigated as bioproduction systems for pharmaceutical proteins for many years. Because the plant shows some differences in protein post-translational modification, many investigators have expressed concern that these differences may cause problems. Our research has shown that plant-derived mIL-12 exhibits similar bioactivity to commercially available recombinant mIL-12 *in vitro*. We are in the process of investigating its *in vivo* activity by using an intranasal vaccination mouse model. Our preliminary data suggest that plant-derived mIL-12 is biologically active *in vivo*, the mouse is tolerant to therapeutic dosages of plant-derived mIL-12 (see Chapter Ⅲ), and that plant-derived mIL-12 is not immunogenic in mice. Thus, our work supports the potential of plant-based systems to produce IL-12 as a pharmaceutical protein.

Ⅱ.5 References

Anderson R, Macdonald I, Corbett T et al. Construction and biological characterization of an interleukin-12 fusion protein (Flexi-12): delivery to acute myeloid leukemic blasts using adeno-associated virus. Hum Gene Ther. 1997;8:1125-1135.

Becker D. Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. 1990;18:203.

Boyer JD, Cohen AD, Ugen KE et al. Therapeutic immunization of HIV-infected chimpanzees using HIV-1 plasmid antigens and interleukin-12 expressing plasmids. AIDS. 2000;14:1515-1522.

Carrington JC, Freed DD. Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. J Virol. 1990;64:1590-1597.

Chen H, Nelson RS, Sherwood JL. Enhanced recovery of transformants of Agrobacterium tumefaciens after freeze-thaw transformation and drug selection. Biotechniques. 1994;16:664-8, 670.

Colombo MP, Trinchieri G. MD:IL-12/ Immunomodulator & adjuvantInterleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002;13:155-168.

Cramer CL, Boothe JG, Oishi KK. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr Top Microbiol Immunol. 1999;240:95-118.

Foss DL, Moody MD, Murphy KP, Jr. et al. *In vitro* and *in vivo* bioactivity of single-chain interleukin-12. Scand J Immunol. 1999;50:596-604.

Galarza JM, Latham T, Cupo A. Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol. 2005;18:365-372.

Gately MK, Brunda MJ. Interleukin-12: a pivotal regulator of cell-mediated immunity. Cancer Treat Res. 1995;80:341-366.

Gorbachev AV, DiIulio NA, Fairchild RL. IL-12 augments CD8+ T cell development for contact hypersensitivity responses and circumvents anti-CD154 antibody-mediated inhibition. J Immunol. 2001;167:156-162.

Gubler U, Chua AO, Schoenhaut DS et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc Natl Acad Sci U S A. 1991;88:4143-4147.

Gutierrez-Ortega A, Avila-Moreno F, Saucedo-Arias LJ, Sanchez-Torres C, Gomez-Lim MA. Expression of a single-chain human interleukin-12 gene in transgenic tobacco plants and functional studies. Biotechnol Bioeng. 2004;85:734-740.

Gutierrez-Ortega A, Sandoval-Montes C, Olivera-Flores TJ, Santos-Argumedo L, Gomez-Lim MA. Expression of functional interleukin-12 from mouse in transgenic tomato plants. Transgenic Res. 2005;14:877-885.

Iturriaga G, Jefferson RA, Bevan MW. Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. Plant Cell. 1989;1:381-390.

Jiang C, Magee DM, Cox RA. Construction of a single-chain interleukin-12-expressing retroviral vector and its application in cytokine gene therapy against experimental coccidioidomycosis. Infect Immun. 1999;67:2996-3001.

Kobayashi M, Fitz L, Ryan M et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med. 1989;170:827-845.

Kwon TH, Seo JE, Kim J et al. Expression and secretion of the heterodimeric protein interleukin-12 in plant cell suspension culture. Biotechnol Bioeng. 2003;81:870-875.

Lam E, Benfey PN, Gilmartin PM, Fang RX, Chua NH. Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. Proc Natl Acad Sci U S A. 1989;86:7890-7894.

Leutenegger CM, Boretti FS, Mislin CN et al. Immunization of cats against feline immunodeficiency virus (FIV) infection by using minimalistic immunogenic defined gene expression vector vaccines expressing FIV gp140 alone or with feline interleukin-12 (IL-12), IL-16, or a CpG motif. J Virol. 2000;74:10447-10457.

Lieschke GJ, Rao PK, Gately MK, Mulligan RC. Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity *in vivo*. Nat Biotechnol. 1997;15:35-40.

Mattner F, Fischer S, Guckes S et al. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. Eur J Immunol. 1993;23:2202-2208.

Medina-Bolivar F, Wright R, Funk V et al. A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine. 2003;21:997-1005.

Medina-Bolivar F, Cramer C. Production of recombinant proteins by hairy roots cultured in plastic sleeve bioreactors. Methods Mol Biol. 2004;267:351-363.

Noguchi Y, Richards EC, Chen YT, Old LJ. Influence of interleukin 12 on p53 peptide vaccination against established Meth A sarcoma. Proc Natl Acad Sci U S A. 1995;92:2219-2223.

Salem ML, Gillanders WE, Kadima AN et al. Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. J Interferon Cytokine Res. 2006;26:593-608.

Shanks JV, Morgan J. Plant 'hairy root' culture. Curr Opin Biotechnol. 1999;10:151-155.

Sin JI, Kim JJ, Arnold RL et al. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell-mediated protective immunity against herpes simplex virus-2 challenge
3. J Immunol. 1999;162:2912-2921.

Tretter V, Altmann F, Marz L. Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase F cannot release glycans with fucose attached alpha 1----3 to the asparagine-linked N-acetylglucosamine residue. Eur J Biochem. 1991;199:647-652.

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood. 1994;84:4008-4027.

Wolf SF, Temple PA, Kobayashi M et al. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J Immunol. 1991;146:3074-3081.

Zagozdzon R, Golab J, Mucha K, Foroncewicz B, Jakobisiak M. Potentiation of antitumor effects of IL-12 in combination with paclitaxel in murine melanoma model *in vivo*. Int J Mol Med. 1999;4:645-648.

Chapter Ⅲ

Intranasal Delivery of Plant-derived IL-12 Enhances Th1 Immunity

Ⅲ.1 Abstract

As a strong immuno-modulator, IL-12 has been investigated as an adjuvant and shown to enhance Th1 immunity in many animal models and clinical trials. In the previous chapter, it was demonstrated that transgenic plants can produce bioactive murine IL-12. Purified plant-derived IL-12 showed equivalent *in vitro* activity as animal-cell-derived IL-12 in inducing interferon-gamma production in cultured lymphocytes and stimulating cell proliferation. To verify whether plant-derived IL-12 is also functional *in vivo*, we investigated a mouse intranasal immunization model utilizing GFP as a model antigen. Although plant-derived IL-12 as well as animal-cell-derived IL-12 did not show adjuvanticity alone, they enhanced the antigen-specific IgG_{2a} level, a marker for Th1 immunity, when co-administered with GFP and the mucosal adjuvant cholera toxin. However, the trial design did not facilitate assessment of the full range of Th1 vs Th2 immune responses. Based on these results, mouse immunization strategies have been proposed for the next IL-12 mouse trial. We also confirmed that plant-derived IL-12 is not immunogenic in mouse. In conclusion, plant-derived murine IL-12 functioned similar to animal-cell-derived IL-12 in enhancing antigen-specific IgG2a levels, suggesting *in vivo* bioactivity in enhancing Th1 immunity.

Ⅲ.2 Introduction

IL-12 is an important immuno-stimulator that enhances the maturation and proliferation of Th1 cells and natural killer (NK) cells. It also enhances the cytotoxicity of cytotoxic T lymphocytes (CTL). IL-12 has been tested as an anti-tumor therapeutic and vaccine adjuvant for cancer and anti-viral vaccines, and showed great promise (reviewed by Colombo and Trinchieri, 2002). However, clinical application of IL-12 has been curbed by the severe side-effects associated with systemic administration (reviewed by Car et al., 1999). Some investigations have suggested that localized administration of IL-12 alleviates the side-effects but retains full anti-tumor activities (reviewed by Salem et al., 2006). IL-12 has also been exploited as a mucosal adjuvant. When intranasally administered with influenza antigen (H1N1) into mice, IL-12 enhanced protective mucosal immunity and increased survival upon lethal virus challenge (Arulanandam et al., 1999; 2001). Salem et al. (2004) found that sustained paracrine-release of IL-12 showed a much lower systemic level of IL-12 but still induced the generation of antigen-specific memory T cells. IL-12 also enhanced Th1 immunities when administered with Th2-biased mucosal adjuvants, such as cholera toxin, and accelerated protective immunity (Albu et al., 2003).

In Chapter 2, it was shown that transgenic plants and hairy roots cultures are capable of efficient bioproduction of the single chain form of murine IL-12 (mIL-12). The 70kDa mIL-12 product produced in these plant tissues cross-reacted with a p70 "conformational" monoclonal antibody specific for mIL-12 in which the p35 and p40 subunits are in the appropriate configuration for bioactivity. Plant-derived IL-12 was purified from cultured transgenic hairy roots by a four-step protein purification scheme. Purified plant-derived mIL-12 stimulated the secretion of IFN-γ from mouse splenocytes and enhanced the proliferation of mouse splenocytes, showing equivalent murine IL-12 bioactivity to animal-cell-derived mIL-12 *in vitro*.

Precursor T cells differentiate into Th1 cells or Th2 cells depending on the manner and environment in which the precursors are stimulated, and IL-12 stimulates the maturation of Th1 cells (reviewed by Abbas et al., 1996; Spellberg et al., 2001). Th1 cells secret IL-2, IL-12, IFN-γ and TNF-α, while Th2 cells produce IL-4, IL-5, IL-10 and IL-13. It has also been reported that B cells produce IgG_{2a} upon interaction with Th1 cells but produce IgG_1 and IgE when interacting with Th2 cells (Fig.Ⅲ.1).

The goal of the current investigation is to test the efficacy of our plant-derived mIL-12 in directing the signature IL-12-mediated shift toward Th1 immunity in an *in vivo* mouse vaccination trial. For these analyses, we utilized a previously developed mouse intranasal vaccination protocol involving GFP (green fluorescent protein) as a model antigen and cholera toxin (CT) as a classic mucosal adjuvant providing a strong Th2-biased immunity (Medina-Bolivar et al., 2003). Cholera toxin (CT) is produced by *Vibrio cholerae*. It acts as an ADP-ribosylase of G proteins and activates adenylate cyclase, which results in a loss of water and chloride in intestinal cells causing diarrhea (Field et al., 1989). CT is widely used in experimental systems as a mucosal adjuvant because it elicits significant systemic and mucosal antibody responses to co-administrated antigens (Elson and Ealding, 1984). Although CT showed great adjuvanticity in animal models, there is little chance for CT to be approved for human healthcare due to its toxicity. Some forms of mutated cholera toxin with reduced or no ADP-ribosylase activity have been generated for potential application as mucosal adjuvants in humans. Mutant CT S61F with little ADP-ribosylase activity enhanced a Th2-biased immune response to co-administered antigen and showed similar adjuvanticity to wild type CT (Yamamoto et al., 1997).

In this chapter, several intranasal immunization experiments were designed to investigate whether plant-derived mIL-12 as well as commercially available animal-cell-derived mIL-12 can enhance GFP-specific Th1 immune responses *in vivo* when intranasally co-administered with mucosal

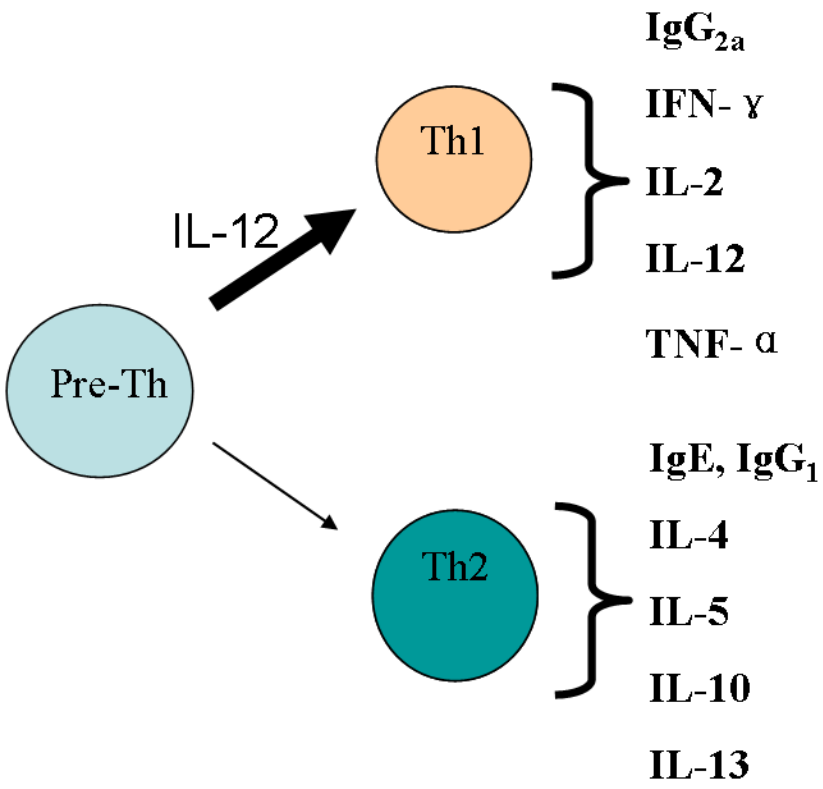


Fig.Ⅲ.1 Precursor T cells differentiate into T helper cells type 1 (Th1) or T helper cells type 2 (Th2) depending on the manner and environment in which the precursors are stimulated. As an important immunomodulator, IL-12 stimulates the differentiation of Th1 cells. Th1 indicators are IgG_{2a}, IFN-γ, IL-2, IL-12 and TNF-α. Th2 indicators include IgE, IgG_1, IL-4, IL-5, IL-10, IL-13. Th: T helper cells

adjuvant CT and model antigen GFP. Selected Th1/Th2 indicators (Fig.Ⅲ.1) were assessed in serum samples and cultured immune responsive cells from immunized mice for the signature IL-12-mediated Th1 shift. An increase antigen-specific IgG_{2a}/IgG_1 ratio in the serum is commonly used as the simplest indicator of a shift toward Th1 immunity (Yamamoto et al., 1997; Lavelle et al., 2001; Albu et al., 2003).

Ⅲ.3 Material and Methods

Ⅲ.3.1 Antigens, adjuvants and cytokines

Plant-derived mIL-12 (pdIL-12) was purified from transgenic tobacco hairy root cultures as described in Chapter 2. Equivalent fractions were collected from non-transgenic hairy roots to serve as control (NT). Animal-cell-derived mIL-12 (acdIL-12) was purchased from Peprotech, Inc. (Chinese hamster ovary cell derived; Rocky Hill, NJ). Murine IL-12 from both sources was standardized by mIL-12 p70 ELISA (R&D Systems, Minneapolis, MN). GFP was purchased from Clonetech, Inc. (Mountain View, CA) and Cholera toxin (CT) from Sigma (St. Louis, MO)

Ⅲ.3.2 Immunization and sera sample collection

Three different mouse immunization experiments (trial 1, 2 and 3) were performed for IL-12 *in vivo* immunological activity investigation. All mice were housed in Arkansas Biosciences Institute Animal Facility at Arkansas State University. The protocol was approved by Arkansas State University Animal Care Committee. Female ICR mice (6-8 week old; 2-6 mice/treatment group depending on experiment) were obtained from Harlan Laboratory and intranasal vaccinations were initiated following a 1-week quarantine. Immunization groups are summarized in Table Ⅲ.1-3 and the immunization/bleeding schedule is summarized in Fig.Ⅲ.2. Briefly mice were lightly anesthetized with 4% isoflurane (Baxter Caribe Inc., Deerfield, IL) utilizing an IMPAC^6 anesthetization chamber (VETEquip, Pleasanton, CA) and immunogen was administered intranasally as a 5 μl aliquot distributed to both nostrils. Administered immunogen included antigen (0.5 μg GFP) and adjuvants as described in Table Ⅲ.1-3, suspended in PBS supplemented with 0.1% normal mouse serum (0.1% NMS/PBS, Sigma). Mice were vaccinated (week 0) and boosted at 2-week intervals (week 2 and 4). For trial 1 and 2, additional mIL-12 treatments were administered intranasally on days 1, 2 and 3 after the primary vaccination and first boost as shown in Fig.Ⅲ.2 following a previously described protocol (Albu et al., 2003). Mice were intranasally immunized with 5 μl aliquots containing 1 μg/mouse/day of

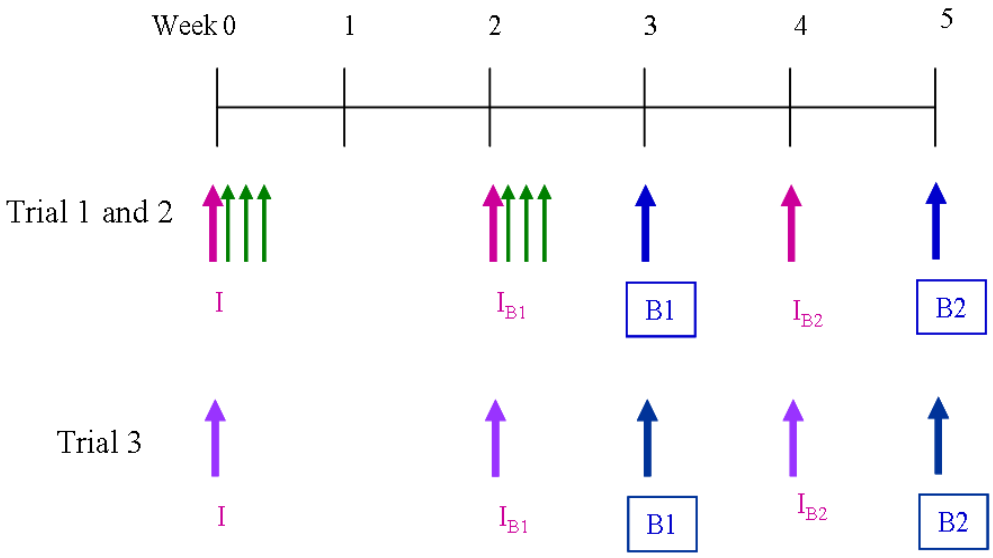


Fig.Ⅲ.2: IL-12 mouse immunization trial schedule for trial 1 to 3.

I Primary immunization admix, IN, under light anesthesia

I_B Secondary immunization admix, IN, under light anesthesia

↑ IL-12 only boosts, IN, under light anesthesia

B Orbital bleed done under light anesthesia

acdIL-12, pdIL-12, or NT control in 0.1% NMS/PBS. At week 3 and week 5, blood was collected followed the orbital bleeding protocol (Fig.Ⅲ.2)

Table Ⅲ. 1: Trial 1 mice immunization group assignment

Group No.	Composition	Number of mice
Group 1	GFP(0.5 μg)	5
Group 2	GFP(0.5 μg) + CT(1.0 μg)	5
Group 3(L)	GFP(0.5 μg) + acdIL-12(0.25 μg)*	2
Group 3 (H)	GFP(0.5 μg) + acdIL-12(1.0 μg)*	3
Group 4 (L)	GFP(0.5 μg) + CT(1.0 μg) + acdIL-12(0.25 μg)*	2
Group 4 (H)	GFP(0.5 μg) + CT(1.0 μg) + acdIL-12(1.0 μg)*	3
Group 5 (L)	GFP(0.5 μg) + pdIL-12(0.25 μg) *	2
Group 5 (H)	GFP(0.5 μg) + pdIL-12(1.0 μg) *	3
Group 6 (L)	GFP(0.5 μg) + CT(1.0 μg) + pdIL-12 (0.25 μg) *	2
Group 6 (H)	GFP(0.5 μg) + CT(1.0 μg) + pdIL-12 (1.0 μg) *	3
Group 7 (L)	GFP(0.5 μg) + CT(1.0 μg) + NT (0.25 μg equivalent)*	2
Group 7 (H)	GFP(0.5 μg) + CT(1.0 μg) + NT (1.0 μg equivalent)*	3

*additional acdIL-12, pdIL-12 or equivalent control from non-transgenic plants were administered for 3 more days (see Fig.Ⅲ.1) at week1 and 3, 0.25 μg or 1.0 μg/mouse/day.

Table Ⅲ.2: Trial 2 mice immunization group assignment

Group No.	Composition	Number of mice
Group 1	GFP(0.5 μg)	6
Group 2	GFP(0.5 μg) + CT(1.0 μg)	6
Group 3	GFP(0.5 μg) + acdIL-12(1.0 μg)*	6
Group 4	GFP(0.5 μg) + CT(1.0 μg) + acdIL-12(1.0 μg)*	6
Group 5	GFP(0.5 μg) + pdIL-12(1.0 μg) *	6
Group 6	GFP(0.5 μg) + CT(1.0 μg) + pdIL-12 (1.0 μg) *	6
Group 7	GFP(0.5 μg) + NT (1.0 μg equivalent) *	6
Group 8	GFP(0.5 μg) + CT(1.0 μg) + NT (1.0 μg equivalent)*	6

*additional acdIL-12, pdIL-12 or equivalent control from non-transgenic plants were administered for 3 more days (see Fig.Ⅲ.1) at week 1 and 3, 1.0 μg/mouse/day.

Table Ⅲ. 3: Trial 3 mice immunization group assignment

Group No.	Composition	Number of mice
Group 1	GFP(0.5 μg) + CT (1.0 μg) +pdIL-12 (1.0 μg)	3
Group 2	GFP(0.5 μg) + CT (1.0 μg) + NT (1.0 μg equivalent)	3

Ⅲ.3.3 Detection of GFP-specific antibody titers

Immulon 4 HBX microtiter plates (Thermo Labsystems) were coated with 100 μl/well of 0.5 μg/ml GFP overnight at room temperature. After blocking with 1%BSA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) for one hour, several dilutions of serum samples were loaded onto the plates (100 μl/well) and incubated for one hour. The plates were washed three time with PBST (0.1% Tween 20 in PBS), 300 μl/well. To measure GFP-specific total serum IgG, horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates Inc., Birmingham, AL) was applied to the plates at 1:5000, 200 μl/well for one hour incubation followed by the addition of HRP substrate (KPL, Inc.) to the plates according to manufacturer's protocols. The plates were then read at 450 nm. GFP-specific IgG titer was calculated by multiplying the absorbance (~0.2) with dilution factor of serum samples.

For subisotyping of IgGs, IgG_1 ELISAs were performed as described above except that HRP-conjugated goat anti-mouse IgG_1 antibody was used for detection of GFP-specific IgG_1. IgG_1 standard and rat anti-mouse IgG_1 monoclonal antibody (Southern Biotechnology Associates Inc., Birmingham, AL) was utilized in the IgG_1 ELISA for quantification of GFP-specific IgG_1 levels. The IgG_{2a} ELISA was the same as IgG_1 ELISA except that HRP-conjugated goat anti-mouse IgG_{2a} antibody was utilized for detection of GFP-specific IgG_{2a}. IgG_{2a} standard and rat anti-mouse IgG_{2a} monoclonal antibody (Southern Biotechnology Associates Inc., Birmingham, AL) were used for IgG_{2a} standard curve.

Ⅲ.3.4 Detection of CT-specific IgG and IL-12-specific IgG

Serum samples were analyzed for the presence of antibodies raised against the adjuvant components, CT and IL-12. Procedures for the CT-specific IgG ELISA and IL-12-specific IgG ELISA were the same as described above for GFP-specific IgG ELISA except that the microtiter plates were coated with 100 μl/well of 1 μg/ml of CT for CT-specific IgG ELISA, and 100 μl/well of 1μg/ml of mIL-12 (Peprotech, Inc.) or plant-derived mIL-12 for IL-12-specific IgG ELISA.

Ⅲ.3.5 Detection of cytokines from GFP-specific cell responses

One week after the final boost, selected mice were sacrificed. Spleens and/or cervical lymph nodes were aseptically removed and pushed through a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ) for single cell suspensions. Isolated cells were cultured at densities of 2.5×10^6 /ml alone, or with mitogen concanavalin A (Sigma; con A, 10 μg/ml), or GFP (25 μg/ml) in culture media (RPMI 1640, 5mM HEPES, 2 mM glutamine, 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 10 mM sodium pyruvate, 1 mM non-essential amino acids). After 3 days culture, the supernatants were collected for Th1/Th2 cytokine assays (Bio-Rad) by using a Bio-Plex suspension array system (Bio-Rad, Hercules, CA) following manufacturer's protocols.

Ⅲ.3.6 Statistics

Results are reported as mean + standard deviation. JMP IN version 4 software (SAS Institute, Cary, NC) was used for statistical analysis. Statistical significance ($P<0.05$) was determined by Means/Anova/t Test.

Ⅲ.4 Results

Ⅲ.4.1 Initial mouse trial for indication of IL-12 immuno-modulating activity *in vivo*

In Chapter 2, we showed that plant-derived mIL-12 (pdIL-12) exhibited similar immuno-stimulating activity *in vitro* to animal-cell-derived mIL-12 (acdIL-12). As IL-12 is a strong immuno-stimulator enhancing Th1 immunity, purified pdIL-12 was compared to acdIL-12 and equivalent purification fractions from non-transgenic plants (NT) in several mouse immunization trials for assessment of IL-12 *in vivo* activities. We designed our immunization regime based on previously described experiments with GFP (model antigen) and CT (model adjuvant) (Medinar-Bolivar et al., 2003), and experiments for intranasal administration of mIL-12 (Albu et al., 2003). AcdIL-12, pdIL-12 and NT control were characterized by silver stain of SDS-PAGE gel (Chapter 2, Fig.Ⅱ.5 A). Th1/Th2 indicators (Fig.Ⅲ.1) from mouse serum samples or cultured immune responsive cells were investigated for possible enhancement of Th1 immunity.

In the initial mouse trial, we investigated IL-12 activity *in vivo* using two different dosages, 0.25μg/mouse or 1μg/mouse. Seven groups of mice (5 mice/group) were immunized with model antigen GFP with or without mucosal adjuvant (CT and/or IL-12 or NT) as shown in Table Ⅲ. 1 at week 0, 2 and 4 (Fig.Ⅲ.2). In group 3-7, 5 mice were divided into two sub-groups with either low dose (L, 0.25 μg IL-12 or equivalent per mouse, 2 mice/subgroup) or high dose (H; 1 μg IL-12 or equivalent per mouse; 3 mice/subgroup) mIL-12. The mice were bled at week 3 and week 5 (Fig.Ⅲ.2) and sera

were collected for detection of anti-GFP IgG. Serum samples from bleed 1 (week 3) showed large variation in anti-GFP IgG titer within each group (data not shown). As a result, we focused on data collected from bleed 2 (week 5). Total GFP-specific IgG titers of bleed 2 are shown in Fig.Ⅲ.3. As shown previously, GFP alone is a poor antigen (Medina-Bolivar et al., 2003). The addition of acdIL-12 or pdIL-12 did not elicit significant secretion of GFP-specific IgG, suggesting that IL-12 alone does not function as a strong adjuvant under these conditions. In contrast, the mucosal adjuvant CT significantly increased the levels of GFP-specific IgG ($P<0.05$; Fig.Ⅲ.3 A). With the presence of IL-12, CT also elicited higher level of GFP-specific IgG than other non-CT groups, although these difference cannot be compared statistically due to small sample size (Fig.Ⅲ.3 A). GFP-specific IgG subisotypes (IgG_1 and IgG_{2a}) were analyzed for group 2, 4, 6 and 7 in which CT was included. Since elevated IgG_{2a} is a marker for Th1 immunity, IgG_{2a}/IgG_1 ratios were used to assess Th1- or Th2-immune responses. Mucosal adjuvant CT alone induced very low ratios of IgG_{2a}/IgG_1 (Fig.Ⅲ.3 B; Table Ⅲ.4). The addition of both acdIL-12 and the high dose of pdIL-12 triggered an increase in the GFP-specific IgG_{2a}/IgG_1 ratio, suggesting stimulation of the Th1 pathway. Although these data appear promising, it should be noted that the equivalent non-transgenic plant extract fractions control also triggered an increase in IgG_{2a}, that the number of mice per group was low and that there was a lot of variability between mice with a group. IL-12 has been linked with toxicity and the additional administrations of IL-12 on days 1, 2, 3 after vaccination was suggested as a mechanism for IL-12 adaptation (Albu et al., 2003). The mice in this trial showed no IL-12 linked weight loss and even the high dose of IL-12 was not linked with any negative health impacts. As a result, we utilized the high dose of IL-12, 1 μg/dose, for all the following IL-12 mouse trials.

Table Ⅲ.4: Subisotype (IgG_1, IgG_{2a}, and IgG_{2a}/IgG_1 ratio) of GFP-specific IgG in trial 1.

Groups	IgG_1 (mg/ml)	IgG_{2a} (mg/ml)	IgG_{2a}/IgG_1 ratio
GFP+CT	55.07 ± 42.66	0.32 ± 0.46	0.0045 ± 0.0067
GFP+CT+acdIL-12 (L)	52.66 ± 42.37	2.20 ± 2.05	0.0386 ± 0.0077
GFP+CT+acdIL-12 (H)	10.23 ± 14.22	0.12 ± 0.16	0.0398 ± 0.0398
GFP+CT+pdIL-12 (L)	33.83 ± 23.86	0.26 ± 0.03	0.0106 ± 0.0083
GFP+CT+pdIL-12 (H)	106.94 ± 69.83	10.32 ± 8.58	0.0870 ± 0.0463
GFP+CT+NT (L)	93.93 ± 96.03	2.01± 2.80	0.0129 ± 0.0166
GFP+CT+NT (H)	255.15 ± 366.36	1.88 ± 2.89	0.0339 ± 0.0530

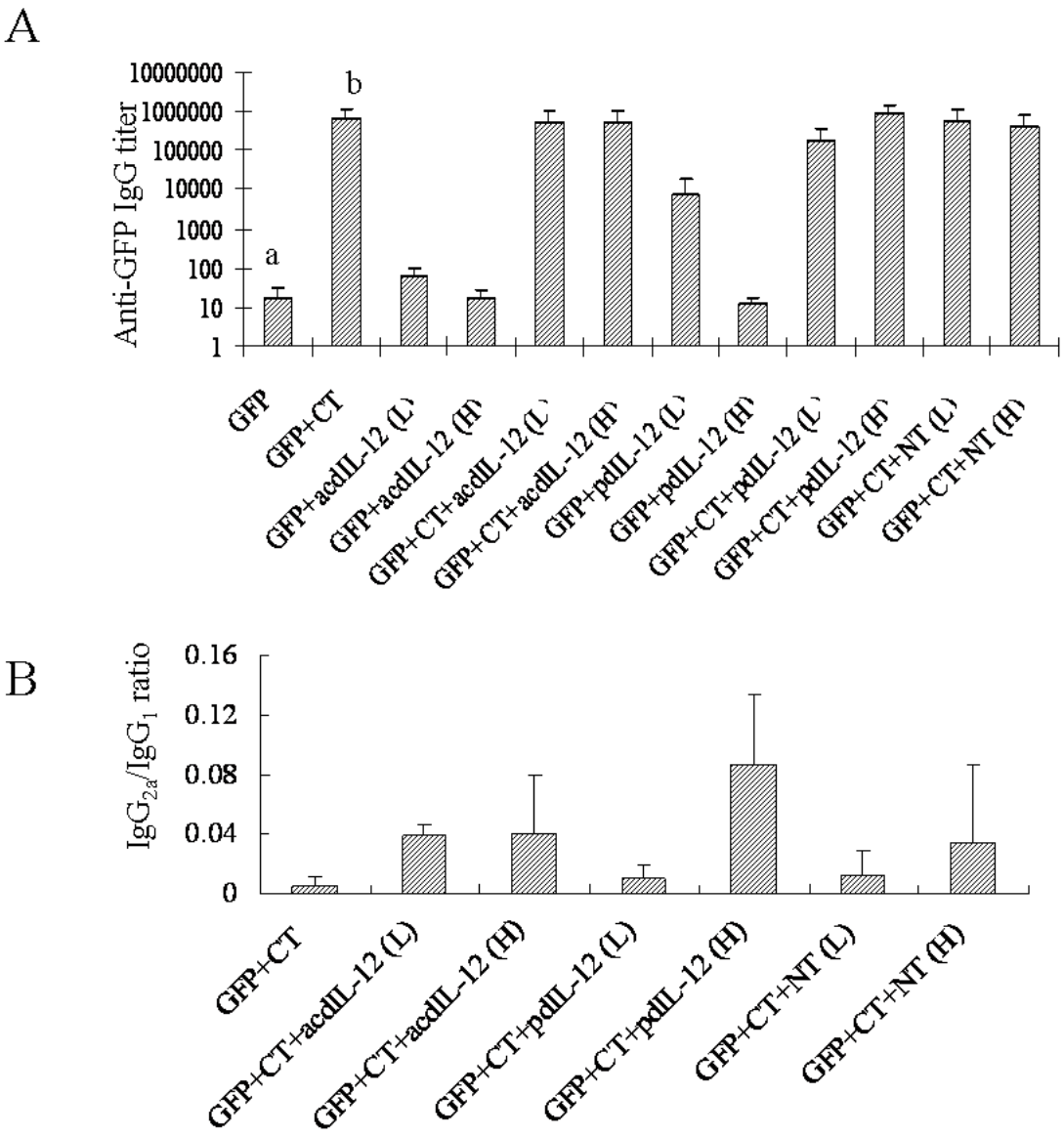


Fig.Ⅲ.3: Serum GFP-specific IgG response determined by ELISA in trial 1. Groups of ICR mice were immunized by GFP alone, or with high dose (H) or low dose (L) of animal-cell-derived IL-12 (acdIL-12), plant-derived IL-12 (pdIL-12), or non-transgenic control (NT), with or without the presence of CT as mucosal adjuvant in week 0, 2 and 4. Data represent sera from bleed 2, collected one week after the final immunization. (A) Total GFP-specific IgG titer. GFP-specific IgG titers were measured as described in Material and Methods. Bars represent the mean IgG titer + 1 SE. (B) Comparison of the ratio of GFP-specific IgG2a/IgG1 in groups immunized with CT. The level of IgG subtype IgG1 and IgG2a were measured by ELISA. IgG2a/IgG1 ratios for individual mice were calculated. Bars represent the mean IgG2a/IgG1 ratio +1 SE. a, b: $p<0.05$, n=5. Sample sizes of the remaining groups were too small to permit statistical analyses

An IL-12-mediated shift toward Th1 responses should also be reflected in antigen-responsive lymphocytes. As IL-12 enhances the maturation of Th1 cells, it was predicted that GFP should stimulate more production of Th1-specific cytokines (e.g. IFN-γ) in cells insolated from the spleens and draining lymph nodes of IL-12 vaccinated mice. For group 2, 4, 6 and 7, spleen cells from 3 mice that showed high GFP-specific IgG responses were isolated and pooled for culture one week after the final booster. Splenocytes were cultured in media with the presence of mitogen (Con A) or GFP for three days before the supernatants were collected for cytokine profile assays. Eight cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, GM-CSF, IFN-γ and TNF-α) that are indicators of Th1, Th2 or undifferentiated Th cells were investigated in these assays. As expected, Con A stimulated the secretion of all cytokines (data not shown). In contrast, the antigen GFP stimulated very low production of IL-4, IL-5, IL-10, IL-12, TNF-α. It is difficult to assess Th1 vs Th2 responses based on these low levels of cytokines. GFP stimulated high levels of a signature Th1 cytokine IFN-γ. As shown in Fig.Ⅲ.4, the addition of acdIL-12 or pdIL-12 (group 4 or 6) enhanced the Th1 cytokine IFN-γ compared to group 2 (GFP+CT), suggesting that acdIL-12 as well as pdIL-12 enhances Th1 immunity when co- administered with CT. However, the NT control group showed the highest level of IFN-γ among all four groups (Fig.Ⅲ.4). This may due to contaminants from plant background as many mitogens are from plants (e.g. Con A, PHA are plant lectin). The high level of IFN-γ in NT control group could also be secreted by other type of immune responsive cells, like NK cells or macrophages. These studies suggest that cell populations enriched from antigen-responsive cells will be required to demonstrate IL-12-mediated induction of Th1 responses. Possibilities include isolation of lymphocytes from the draining lymph nodes or enrichment of specific splenocytes population via cell-sorting or antibody/magnetic bead enrichment protocols.

Ⅲ.4.2 The second mouse trial on IL-12 immuno-modulating activity *in vivo*

Our initial vaccine trial suggested that plant-derived IL-12 mediated a shift toward Th1 immunity and warranted a second trial to establish statistically relevant data. In trial 1, our results suggested that high dose of IL-12 (1 μg/dose) is more effective in enhancing Th1 immunity than the low dose one. As a result, we utilized 1 μg/dose of IL-12 in trial 2. Thus, 8 groups of mice (6 mice/group) were immunized with model antigen GFP with or without mucosal adjuvant (CT and/or IL-12 or NT) as shown in Table Ⅲ.2 at week 0, 2 and 4 (Fig.Ⅲ.2). Blood serum samples collected at week 5 were analyzed for GFP-specific IgG and IgG subisotypes (IgG_1 and IgG_{2a}). As shown in trial 1, the addition of acdIL-12, pdIL-12, or NT did not elicit high level of GFP-specific IgG, but the presence

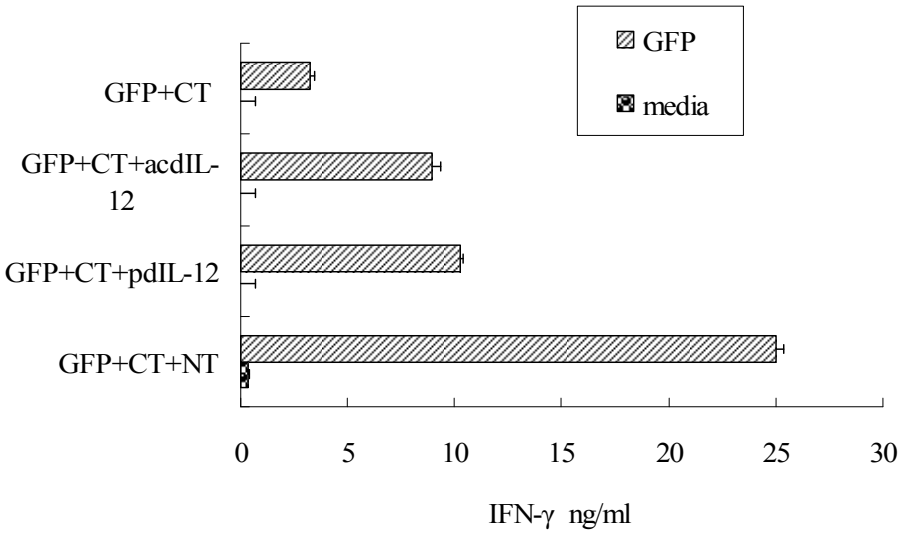


Fig.Ⅲ.4: Interferon-gamma (IFN-ɣ) secretion from cultured spleen cells from mice vaccinated in trial 1. Splenocytes were isolated from groups of ICR mice immunized with GFP in the presence of CT, with or without animal-cell-derived IL-12 (acdIL-12), plant-derived IL-12 (pdIL-12), or non-transgenic control (NT). One week after the final immunization, three mice from each group were sacrificed and spleen cells were isolated, pooled and cultured with (hatched bars) or without (sphere bars) GFP (25 μg/ml) for 3 days. Cell culture supernatants of were collected and assayed for IFN-ɣ levels. Bars represent the mean level of cytokines +1 SE of duplicates.

of CT dramatically enhanced anti-GFP IgG level in all CT treatment groups (Fig.Ⅲ.5 A). Assessment of IgG subisotypes showed an increase in IgG_{2a}/IgG_1 ratios in the presence of either acdIL-12 or pdIL-12 (Fig.Ⅲ.5 B; Table Ⅲ.5). Our results confirmed that pdIL-12 showed similar *in vivo* activity in enhancing Th1 immunity compared to acdIL-12. However, in the second mouse trial, GFP-specific IgG titers from all CT groups were all below 1×10^5 (Fig.Ⅲ.5 A), and these titers are much less than those from the first trial (1×10^5 to 1×10^6; Fig.Ⅲ.3 A). Many mice among CT treated groups (1-4 mice/group) did not show significant GFP-specific IgG responses (non-responders). The reduced and highly variable responses may be due to several factors that impacted this trial. At approximately 10 weeks of age, these mice on average were about 10 grams heavier than those from the first trial (trial 1: ~30 g; trial 2:~40 g) and showed much greater variability in weight (data not shown). Two weeks after the mouse trial started, we found some mice had ear infections and the infection spread to most of the mice as the trial processed. Antibiotic ointment was applied to all the infected mice to control the infection. Due to the problems experienced by the mice in this trial, we will have to repeat this mouse trial before we draw any definitive conclusions.

Table Ⅲ.5: Subisotype (IgG_1, IgG_{2a}, and IgG_{2a}/IgG_1 ratio) of GFP-specific IgG in trial 2.

Groups	IgG_1 (mg/ml)	IgG_{2a} (mg/ml)	IgG_{2a}/IgG_1 ratio
GFP+CT	24.04 ± 29.92	0.03 ± 0.02	0.0021 ± 0.0031
GFP+CT+cIL-12	211.37 ± 143.56	0.82 ± 0.80	0.0057 ± 0.0072
GFP+CT+pdIL-12	56.01 ± 77.16	1.16 ± 0.90	0.0143 ± 0.0035
GFP+CT+NT	44.75 ± 80.36	0.15	0.0004 ± 0.0005

Therefore, while the second trial was again suggestive of IL-12-mediated induction of Th1 responses and comparable responses between the animal-derived and plant-derived cytokines, the heterogeneity of mice due to weight differences and background infections undermine the utility of this data in demonstrating *in vivo* equivalency. However, because of these mice exhibited some increases in GFP-specific immunity, spleens and draining lymph nodes (cervical lymph nodes) were harvested for developing protocols for assessing Th1 versus Th2 cytokine responses (see Appendix).

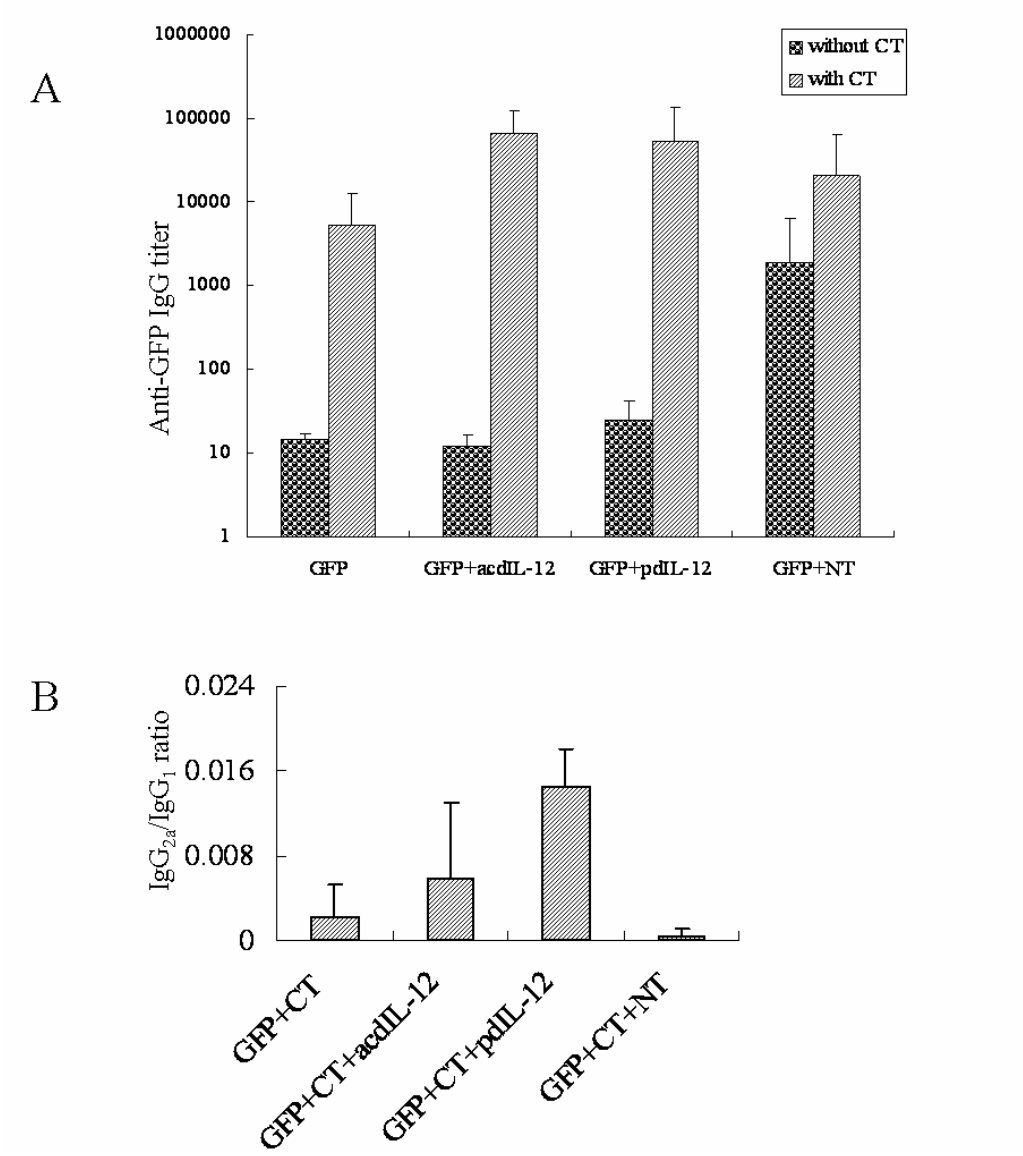


Fig.Ⅲ.5: Serum GFP-specific IgG responses determined by ELISA in trial 2. Groups of ICR mice were immunized by GFP alone, or with animal-cell-derived IL-12 (acdIL-12), plant-derived IL-12 (pdIL-12), or equivalent non-transgenic control fractions (NT), in the presence (hatched bars) or absence (sphere bars) of CT as mucosal adjuvant in week 0, 2 and 4. Serum samples were collected one week after the final immunization. (A) Comparison of GFP-specific IgG titer. GFP-specific IgG titers were measured as described in Material and Methods. Bars represent the mean IgG titer + 1 SE. (B) Comparison of the ratio of GFP-specific IgG_{2a}/IgG_1 in groups immunized with CT. The level of IgG subisotype IgG_1 and IgG_{2a} were measured by ELISA. IgG_{2a}/IgG_1 ratios for individual mice were calculated. Bars represent the mean IgG_{2a}/IgG_1 ratio +1 SE.

Ⅲ.4.3 Assessment of IL-12 treatment regimes in the third mouse trial

In trial 1 and trial 2, we used 4-day consecutive administration of IL-12 to reduce potential toxicity (Albu et al., 2003). However, this protocol involves extensive manipulations of the animals and is unlikely to be clinically acceptable. In order to study whether we can reduce the administration of IL-12 from 4 days to 1 day in each vaccination, we initiated a preliminary experiment with two groups of mice (3 mice/group, Table Ⅲ.3). These mice were immunized with GFP and CT supplemented with 1μg pdIL-12 or equivalent fractions from NT controls at week 0, 2 and 4 (Fig.Ⅲ.2; Trial 3). Blood serum samples collected at week 5 were analyzed for GFP-specific IgG and IgG subisotypes. Our results showed that these mice exhibited high level of anti-GFP IgG in their blood sera and pdIL-12 enhanced the IgG_{2a}/IgG_1 ratio (Fig.Ⅲ.6; Table Ⅲ.6), though the increase of IgG_{2a}/IgG_1 ratio was not as dramatic as what we have shown in the first and second trial with 4-day consecutive administration of IL-12 (Fig.Ⅲ.3 B, 5 B and 6 B; Table Ⅲ.4; Table Ⅲ.5; Table Ⅲ.6).

Table Ⅲ.6: Subisotype (IgG_1, IgG_{2a}, and IgG_{2a}/IgG_1 ratio) of GFP-specific IgG in trial 3.

Groups	IgG_1 (mg/ml)	IgG_{2a} (mg/ml)	IgG_{2a}/IgG_1 ratio
GFP+CT+pdIL-12	247.58 ± 291.09	0.76 ± 0.96	0.0026 ± 0.0004
GFP+CT+NT	636.80 ± 627.01	0.98 ± 1.36	0.001 ± 0.0008

This trial involved the same shipment of mice as trial 2 and displayed similar issues of size heterogeneity and highly variable immune responses. Thus, additional experiments are required to determine the effectiveness of this reduced IL-12 administration regime in eliciting Th1 immunity. However, no side-effects, or signs of toxicity were observed among these mice. Our results suggest that it may not be necessary to administer extra IL-12 on day 2, 3 and 4 for IL-12 to show its *in vivo* activity without toxicity. Spleen cells from these mice were collected for protocol development including the isolation of CD4+ T (T helper) cells. These enriched Th cells were tested for possible IL-12-mediated Th1 shift (Appendix).

Ⅲ.4.4 Immunogenicity of IL-12

IL-12 is a glycoprotein and N-linked glycosylation is important for bioactivity (Carra et al., 2000). There have been concerns that differences in sugar composition of N-linked glycans in recombinant proteins produced in heterologous system may trigger immune responses to the recombinant protein or to its endogenous homolog. Plant-derived IL-12 probably has different N-

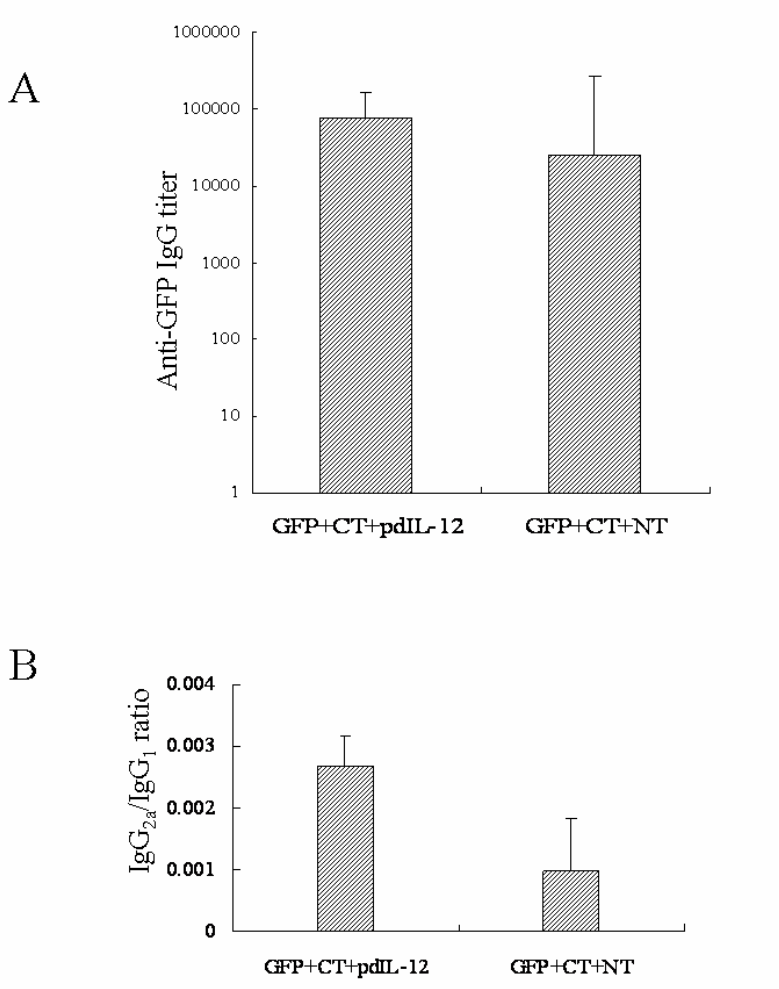


Fig.Ⅲ.6: Serum GFP-specific IgG response determined by ELISA in trial 3. Two groups of ICR mice were immunized by GFP, CT, and plant-derived IL-12 (pdIL-12) or non-transgenic control (NT) at week 0,2 and 4. Mouse blood sera were collected one week after the final immunization. (A) Comparison of GFP-specific IgG titer. GFP-specific IgG titers were measured as described in Material and Methods. Bars represent the mean IgG titer + 1 SE. (B) Comparison of the ratio of GFP-specific IgG_{2a}/IgG_1 in groups immunized with CT. The level of IgG subisotype IgG_1 and IgG_{2a} were measured by ELISA. IgG_{2a}/IgG_1 ratios for individual mice were calculated. Bars represent the mean IgG_{2a}/IgG_1 ratio +1 SE.

glycosylation from animal-cell-derived IL-12 (see Chapter 2). To test whether the different glycosylation of pdIL-12 would cause immunogenicity in mice, mouse sera collected from the second mouse trial were analyzed by IL-12-specific IgG ELISA. The results showed that neither acdIL-12 nor pdIL-12 triggered strong acdIL-12-specific IgG immune responses, i.e. using acdIL-12 as the capture antigen for IgG ELISA (Fig.Ⅲ.7). Sera samples collected from group 6 (GFP+CT+pdIL-12) also did not show high level of anti-pdIL-12 IgG, i.e. using the pdIL-12 as capture antigen for IgG ELISA (data not shown), suggesting plant-derived IL-12 was not immunogenic in mice. On the contrary, the CT-specific IgG ELISAs of mouse serum samples suggest CT itself has high immunogenicity (Fig.Ⅲ.7), and this result is consistent with what has been previously reported about the immunogenicity of CT (Elson and Ealding, 1984). Although CT mediated high IgG responses to the poor immunogen GFP, IgG responses against acdIL-12 or pdIL-12 were not elicited in the presence of this strong mucosal adjuvant (Fig.Ⅲ.7). These results suggest that the three-dimensional conformation of both plant-derived single chain form and CHO-derived heterodimer are sufficiently similar to the native mIL-12 to be recognized as "self" rather than "foreign" even in the presence of the strong adjuvant CT.

Ⅲ.5 Discussion

Our goal in these experiments was to demonstrate that mIL-12 synthesized in plants showed equivalent *in vivo* immuno-modulating activity as animal-cell-derived IL-12 leading to a "signature" Th1-biased immunity. Neither pdIL-12 nor acdIL-12 when administered alone with the poorly immunogenic model antigen GFP, functioned as a strong adjuvant in enhancing GFP-specific antibody responses under these intranasal vaccination protocols. However, both mIL-12s, when administered intranasally with GFP and the mucosal adjuvant CT, increased the IgG_{2a}/IgG_1 ratio. Although the difference is not significant due to small sample size in trial 1 and unexpected mouse issues in trial 2, our results still suggest a Th1-enhanced immunity in mice administered with either acdIL-12 or pdIL-12. These trials need to be expanded and additional indicators of Th1 versus Th2 immunity need to be assayed in order to establish bioequivalency in Th1 immuno-modulating activity (see below). Interestingly, mice immunized with GFP and CT with either plant- or animal-cell-derived IL-12 raised strong antibody titers to GFP and to CT, but not to IL-12. This suggests that the recombinant IL-12 glycoprotein produced in plants is sufficiently mIL-12-like to be non-immunogenic. This is an important finding as one considers using plant production systems for producing human IL-12 for vaccination applications.

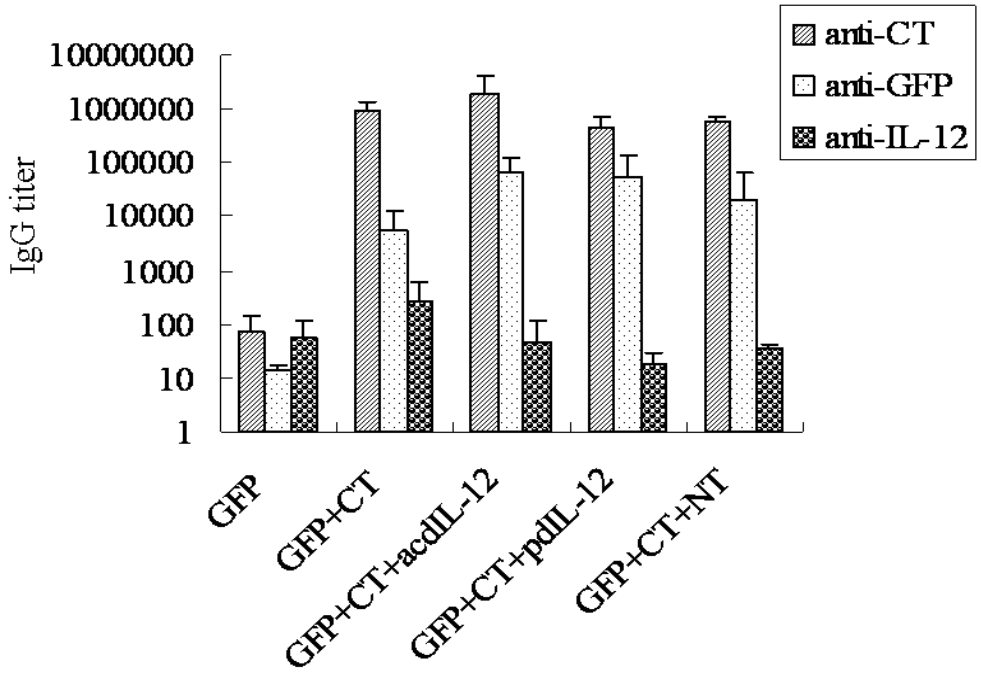


Fig.Ⅲ.7: Serum CT- and IL-12-specific IgG responses in trial 2. Groups of ICR mice were immunized by GFP alone, or with the presence of CT, with or without animal-cell-derived IL-12 (acdIL-12), plant-derived IL-12 (pdIL-12), or non-transgenic control (NT) in week 1,3 and 5. Mouse blood sera were collected one week after the final immunization. CT-specific IgG titer (hatched bars) were measured by CT-specific IgG ELISA and IL-12-specific IgG titers (sphere bars) were determined by IL-12-specific IgG ELISA as described in Material and Methods. GFP-specific IgG titers (dotted bars) were shown as control (Fig.Ⅲ.5A). Anti-IL-12 results shown are those using acdIL-12 as capture protein for ELISA. Analogous results were obtained fro ELISAs using pdIL-12 as capture protein. Bars represent the mean IgG titer +1 SE in each group.

Although the vaccine trials presented in this chapter were not definitive in demonstrating the efficacy of pdIL-12 (or acdIL-12) in leveraging a Th1-biased immune response, they identified a number of factors that can be altered or modified in order to design a more effective vaccination protocol. These include choice of mouse strain, choice of antigen, IL-12 administration regime, and systems for Th1/Th2 immunity readout. These are addressed briefly below.

Mouse strain: The current trial utilized the outbred ICR mice since this strain showed higher IgG responses to GFP when administer with CT or as a fusion with the ricin B subunit than several inbred strains (Medina-Bolivar et al., 2003; Dolan, Wright and Cramer, unpublished). Using inbred strains (e.g. C57BL/6 or Balb/c) should reduce the inter-animal variability of immune responses. Both C57BL/6 and Balb/c are widely used in vaccination trials often with ovalbumin (OVA) as the model antigen (Yamamoto et al., 1997; Lavelle et al., 2001). C57BL/6 is the most widely used inbred strain and was the DNA source for the first high quality draft sequence of the mouse genome (Jackson Laboratory). Balb/c mice demonstrate Th2-biased immune responses (Jackson Laboratory). Thus, I would recommend selecting one of these 2 inbred strains for the next trial and increasing the number of mice per group to facilitate statistical analyses if necessary.

Antigen: GFP was selected as the model antigen in order to build on our experience with previous vaccine trials. For these trials the choice of GFP was based on its utility as a fusion partner for ricin B, permitting detection in cell uptake experiments. GFP is not a widely used antigen in vaccine research and is relatively expensive. The widely used OVA may provide a better model antigen for these experiments. Many studies described OVA-specific immunogenicity using a broad array of adjuvants and routes of administration (Lavelle et al., 2001; Kunisawa et al., 2000; Cho et al., 2000). Alternatively, well-defined protective antigens for infectious disease models may be advantageous, permitting assessment of immunity to include protection against pathogen challenge in addition to assessment of Th1 vs Th2-enhanced cytokine and antibody profiles.

IL-12 administration regime: We utilized a 4-day consecutive administration of IL-12 to reduce potential toxicity of IL-12 (Leonard et al., 1997; Albu et al., 2003). However, 4-day administration of IL-12 is troublesome, time-consuming and probably is unacceptable for clinical applications. In order to develop a better regime for IL-12 mouse vaccination, we initiated a pilot trial for 1-day administration of IL-12. Our preliminary data suggest that 1-day administration also triggered higher level of IgG2a, although the increase was not as substantial as the 4-day regime. Because the primary goal of the next trial is to establish IL-12 efficacy and bioequivalency, it may be best to use the 4-day

administration regime. However, further studies may target the development of better regimes to reduce the 4-day administration.

Readouts on Th1 vs Th2 immunity: We showed that IL-12 (acdIL-12 and pdIL-12) increased the IgG2a level in the blood serum, suggesting Th1-enhanced immunity. To investigate whether IL-12 can enhance the maturation of antigen-specific Th1 cells, which would be beneficial to anti-tumor and anti-viral vaccines, we tried to utilize cytokine profiling of spleen cells and lymph node cells isolated from immunized mice for antigen-specific cell responses. Although both Th1 and Th2 cytokines could be detected in these systems (see Appendix), our data were not clear in showing a Th1- or Th2-enhanced immunity in IL-12 mouse groups. There are several options to improve the readouts on Th1/Th2 immunities. One will be to collaborate with an immunology laboratory and use FACS (Fluorescence associated cell sorting). We can label spleen cells or lymph node cells isolated from immunized mice with fluorescence-labeled antigen, Th1 cytokine (e.g. IFN-g) antibodies and Th2 cytokine (e.g. IL-5) antibodies, so that it can be directly detected if there is more Th1 cells expressing antigen-specific T cell receptors in IL-12 group. Another strategy will be to utilize enriched CD4+ T cells from spleen cells for cytokine profiling test. We tested CD4+ cells enrichment strategies with splenocytes from mice in vaccination trial 3. Preliminary studies suggested that enriched CD4+ T cells provide higher antigen-specific responses in the presence of a co-stimulant anti-CD28 antibody (see Appendix). Thus, this appears to be a better strategy for assessing cytokine responses.

Purification of pdIL-12: Our results suggest that there may be some contaminants in the equivalent fractions from non-transgenic controls triggering immune responses. This problem may be solved by improving the purification scheme for pdIL-12. By either developing another step of purification, or narrowing collecting fraction window, it is possible to increase the purity of pdIL-12 and obtain fewer contaminants in the NT control. This probably would reduce the possibility of NT control triggering immune responses.

Interest in IL-12 is focused on its ability to function as an anti-cancer therapeutic or vaccine adjuvant for anti-cancer vaccines or vaccines targeting intracellular pathogens and parasites. Thus, the direct test of plant-derived IL-12 *in vivo* activity would be utilizing it in mouse tumor models and infectious disease models to test whether it shows anti-tumor activity and induces protective immunity. Toward this end, we have recently sent our purified pdIL-12 and NT control to two research groups for IL-12 *in vivo* anti-tumor and anti-viral activity investigations. One research group (Dr. M. Salem, Medical University of South Carolina; Charleston, SC) will investigate whether plant-derived IL-12 shows similar activity to animal-cell-derived IL-12 in a paracrine-releasing system (F2 gel) in an OT-1

mouse model (Salem et al., 2004). The other research group (led by Drs. Mark Tompkins and Ralph Tripp, University of Georgia, Athens, GA) will utilize plant-derived IL-12 in an influenza mouse model to test whether it can enhance protective immunity (Tompkins et al., 2004). These collaborations certainly will help us understand the function of plant-derived IL-12 *in vivo* and may contribute to the commercialization of this recombinant protein.

Ⅲ.6 References

Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996; 383: 787-793.

Albu DI, Jones-Trower A, Woron AM, Stellrecht K, Broder CC, Metzger DW. Intranasal vaccination using interleukin-12 and cholera toxin subunit B as adjuvants to enhance mucosal and systemic immunity to human immunodeficiency virus type 1 glycoproteins. J.Virol. 2003; 77: 5589-5597.

Arulanandam BP, Metzger DW. Modulation of mucosal and systemic immunity by intranasal interleukin 12 delivery. Vaccine 1999; 17: 252-260.

Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against Streptococcus pneumoniae infection 1. Infect.Immun. 2001; 69: 6718-6724.

Car BD, Eng VM, Lipman JM, Anderson TD. The toxicology of interleukin-12: a review. Toxicol.Pathol. 1999; 27: 58-63.

Carra G, Gerosa F, Trinchieri G. Biosynthesis and posttranslational regulation of human IL-12. J.Immunol. 2000; 164: 4752-4761.

Cho HJ, Takabayashi K, Cheng PM et al. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. Nat.Biotechnol. 2000; 18: 509-514.

Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002; 13: 155-168.

Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J.Immunol. 1984; 132: 2736-2741.

Field M, Rao MC, Chang EB. Intestinal electrolyte transport and diarrheal disease (1). N.Engl.J.Med. 1989; 321: 800-806.

Kunisawa J, Okudaira A, Tsutusmi Y et al. Characterization of mucoadhesive microspheres for the induction of mucosal and systemic immune responses. Vaccine 2000; 19: 589-594.

Lavelle EC, Grant G, Pusztai A, Pfuller U, O'Hagan DT. The identification of plant lectins with mucosal adjuvant activity. Immunology 2001; 102: 77-86.

Leonard JP, Sherman ML, Fisher GL et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood 1997; 90: 2541-2548.

Medina-Bolivar F, Wright R, Funk V et al. A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine 2003; 21: 997-1005.

Salem ML, Kadima AN, Zhou Y et al. Paracrine release of IL-12 stimulates IFN-gamma production and dramatically enhances the antigen-specific T cell response after vaccination with a novel peptide-based cancer vaccine. J.Immunol. 2004; 172: 5159-5167.

Salem ML, Gillanders WE, Kadima AN et al. Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. J.Interferon Cytokine Res. 2006; 26: 593-608.

Spellberg B, Edwards JE, Jr. Type 1/Type 2 immunity in infectious diseases. Clin.Infect.Dis. 2001; 32: 76-102.

Tompkins SM, Lo CY, Tumpey TM, Epstein SL. Protection against lethal influenza virus challenge by RNA interference *in vivo*. Proc.Natl.Acad.Sci.U.S.A 2004; 101: 8682-8686.

Yamamoto S, Kiyono H, Yamamoto M et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc.Natl.Acad.Sci.U.S.A 1997; 94: 5267-5272.

Chapter Ⅳ

Lectin Functions as Molecular Carrier to Enhance Mucosal Deliver Efficacy of IL-12

Ⅳ.1 Abstract

The immuno-modulating cytokine, interleukin-12 (IL-12), has great potential as an anti-tumor therapeutic and vaccine adjuvant for cancers and viral infections. However, its clinical application was hindered by severe side-effects associated with systemic administration. Several studies have suggested that mucosal delivery of IL-12 is as effective as systemic administration for immune modulation with much reduced toxicity. Ricin B (RTB), the non-toxic carbohydrate-binding subunit of ricin, is essential to the uptake of ricin into mammalian cells and the intracellular trafficking of ricin A, the catalytic subunit of ricin, from the endomembrane system to the cytosol, where ricin A inactivates ribosomes. RTB's function suggests that it may work as a molecular carrier for effective mucosal delivery of IL-12. To test this hypothesis, transgenic plants producing RTB:IL-12 fusions were generated and characterized. Our results demonstrated that RTB fused to the carboxyl-terminus of IL-12 maintained full lectin activity and IL-12 bioactivity. RTB fused to the amino-terminus of IL-12 did not show lectin activity due to steric hindrance. Purified IL-12:RTB from transgenic plant tissue was tested in an *in vitro* mucosal-associated lymphoid tissue (MALT) assay. The results indicate that RTB facilitates the binding of IL-12 to the epithelial cells and presentation of IL-12 to immune responsive cells.

Ⅳ.2 Introduction

IL-12 is a very important immuno-modulator in that it enhances cell-mediated immunity (CMI) and inflammation. It stimulates the secretion of interferon-gamma (IFN-γ) from T cells and natural killer (NK) cells and activates the innate resistance to infections. It also plays vital roles in the maturation and differentiation of type 1 T helper cells (Th1) and cytotoxic T lymphocytes (CTL) (Trinchieri, 1994; 2003). It shows great potential as an anti-tumor therapeutic and adjuvant for cancer and viral vaccines (reviewed by Colombo and Trinchieri, 2002). However, its clinical application was hindered by severe side-effects associated with systemic administration (Leonard et al., 1997). Localized presentation through subcutaneous gels or intranasal administration has suggested that mucosal delivery may allow for the desired IL-12 efficacy at much lower concentrations and thus greatly reduced toxicity (Huber et al., 2003; Salem et al., 2004).

Ricin, a type II ribosome-inactivating protein (RIP) plant toxin from castor bean (*Ricinus communis*), is a heterodimer consisting of two subunits, ricin A (RTA) and ricin B (RTB). RTA, the catalytic subunit, has *N*-glycosidase activity and inactivates the ribosomes (Endo et al., 1987). RTB, the galactose/galactosamine-binding subunit of ricin, binds to glycan-rich mammalian cell surfaces so

that ricin is internalized into cells through receptor-mediated endocytosis (Mohanraj et al., 1995). Many investigations suggest multiple subcellular transport pathways for ricin following endocytosis (reviewed by Sandvig et al., 2000). Indirect evidence suggests that ricin reaches the endoplasmic reticulum (ER) where RTB is degraded and RTA is translocated to the cytosol by translocons (ER-localized Sec61; Roberts and Smith, 2004). Ricin may also be transported from endosomes to lysosomes and becomes digested (Magnusson et al., 1993), or recycled to the cell surface by transcytosis (van Deurs et al., 1990).

RTB plays important roles in these multiple transport pathways of ricin. The two galactose-binding domains of RTB are essential to the cytotoxicity of ricin (Newton et al., 1992), because it not only facilitates the internalization of ricin into the cells but also aids in the retrograde transport of RTA from the endosomes to the ER (reviewed by Roberts and Smith, 2004). In addition, RTB is a glycoprotein. The mannosylated glycans also interact with the D-mannose receptor on the surface of certain type of cells (such as macrophages) and ricin enters cells via the clathrin-coated pit pathway (Frankel et al., 1997).

The fact that RTB is involved in multiple transport pathways into and within the cell suggests that RTB may have utility as a molecular carrier to facilitate the localized presentation of antigens or cytokines, such as IL-12, to the mucosal immune responsive cells (Fig.Ⅳ.1). Disarmed ricin fused with a small peptide facilitated the presentation of the small peptide to MHC class I molecules, indicating disarmed ricin could be an adjuvant for cancer vaccines (Smith et al., 2002). Medina-Bolivar et al. (2003) found that transgenic tobacco-produced RTB worked as mucosal carrier/adjuvant and mediated the induction of primarily Th2-skewed immune responses to its fusion partner GFP.

Previous research has already shown that transgenic plants are able to produce bioactive RTB lectin and IL-12 cytokine, respectively (Chapter 2; Reidy and Cramer, unpublished; Medinar-Bolivar et al., 2003; Reed et al., 2005; Kwon et al., 2003; Gutierrez-Ortega et al., 2004; 2005). In order to test the potential of RTB to function as a mucosal carrier and fusion partner for IL-12, transgenic plants were generated to produce RTB fused to the single-chain form of murine IL-12 (mIL-12). The lectin activity of mIL-12:RTB fusions was determined by their ability to bind to asialofetuin, a galactose-rich glycoprotein, in a microtiter plate-binding assay (Dawson et al., 1995). Purified mIL-12:RTB fusion products showed both IL-12 biological activity and lectin activity. An *in vitro* mammalian cell culture model was utilized to demonstrate that RTB acts as a molecular carrier and may facilitate the uptake of mIL-12 into mucosal associated lymphoid tissue (MALT).

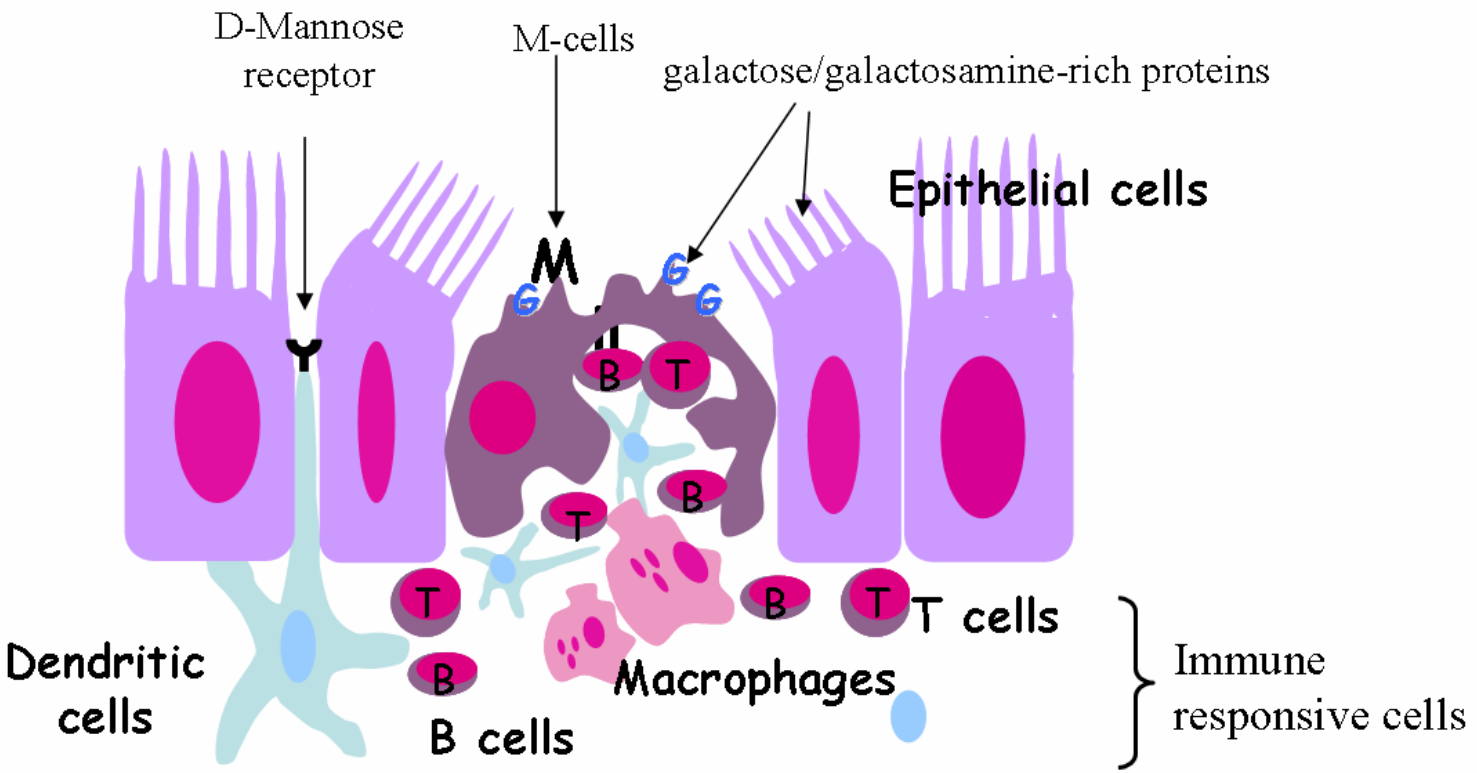


Fig. 1. The structure of mucosal-associated lymphoid tissue (MALT). RTB binds to galactose/galactosamine-rich proteins (G) and D-mannose receptor (Y). This figure was kindly provided by Dr. M. Dolan with minor modification.

Ⅳ.3 Material and methods

Ⅳ.3.1 Construction of *RTB:IL-12* gene fusion

IL-12 sequences encoding the "mature" single chain murine IL-12 (scmIL-12, lacking the p40 signal peptide) were amplified from plasmid pSFG-mIL-12.p40.L.delta.p35 (Lieschke et al., 1997) by PCR using primer 5'-CTCGAGATGTGGGAGCTGGAGAAAG and primer 5'-GAGCTCTCAGGCGGAACTCAGATAG which incorporated flanking restriction enzyme sites (underlined) for *Xho*I and *Sac*I, respectively. A DNA fragment containing the constitutive 35S promoter (double enhanced 35S promoter; Becker, 1990), the TEV translational enhancer (Carrington et al., 1990), and the sequences encoding the patatin signal peptide (pat, Iturriaga et al., 1989) and ricin B subunit 35S:pat:RTB was obtained by digesting plasmid R6-2 (Medina-Bolivar et al., 2003) with *Hind*III and *Xho*I. Plasmid pBC (Stratagene, La Jolla, CA) was digested with *Hin*dIII and *Sac*I and ligated in a tri-molecular reaction with *IL-12* and *35S:pat:RTB* fragments to yield plasmid *RTB:IL-12* (Fig.Ⅳ.2).

Ⅳ.3.2 Construction of *RTB:L:IL-12* plasmid

In order to place a flexible linker between the IL-12 and RTB compounds, a pair of oligonucleotides encoding $(Gly_3Ser)_3$ flanked with *Xho*I restriction sites on both ends (5'-ACGCTCGAGGGAGGTGGATCAGGTGGCGGATCTGGTGGAGGTTCTCTCGAGTAC) was synthesized (MWG-Biotech, High Point, NC). After annealing, the double-stranded oligo was digested by *Xho*I.

RTB:IL-12 construct in pBC (described above) was digested with *Eco*RI and *Sac*I so that the construct was cleaved into two fragments, A and B (Fig.Ⅳ.3). Fragment A (*Eco*RI-RTB:IL-12-*Sst*I) containing *TEV:pat:RTB:IL-12* was inserted into a pBC vector in which *Kpn*I and *Xho*I sites were eliminated by digestion with Mung Bean Nuclease (New England BioLabs, Boston, MA). The consequent construct was then digested by *Xho*I and ligated with the *Xho*I-digested linker fragment. Following sequencing confirmation, fragment *Eco*R I-*RTB:linker:IL-12-Sac* I was digested out and inserted back into fragment B. This construct was called *RTB:L:IL-12* (Fig.Ⅳ.3).

Ⅳ.3.3 Construction of *IL-12:RTB* gene fusion

For construction of the IL-12:RTB plant expression vector, an IL-12 fragment (*Kpn*I-*p40:L:p35*-*Sac*I) without the stop codon was amplified from pSFG-mIL-12.p40.L.delta.p35 (Lieschke

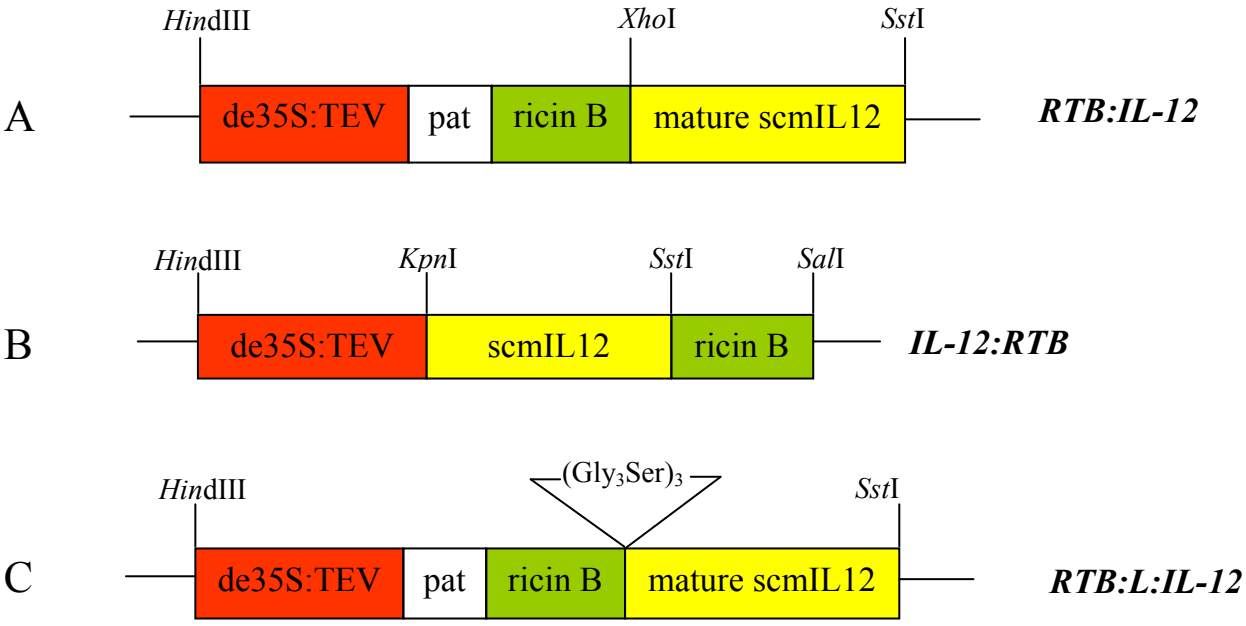


Fig.Ⅳ.2 Constructs generated for expression of RTB:mIL-12 (A), mIL-12:RTB (B) and RTB:L:mIL-12 (C) in transgenic plants. de35S: double enhanced 35S promoter; TEV: translational enhancer; pat: patatin signal peptide; mature scmIL-12: DNA fragment that encoding murine IL-12 (p40-p35) without endogenous signal peptide; scmIL-12: DNA fragment that encoding murine IL-12 with mIL-12 p40 endogenous signal peptide; RTB: DNA fragment encoding ricin B subunit.

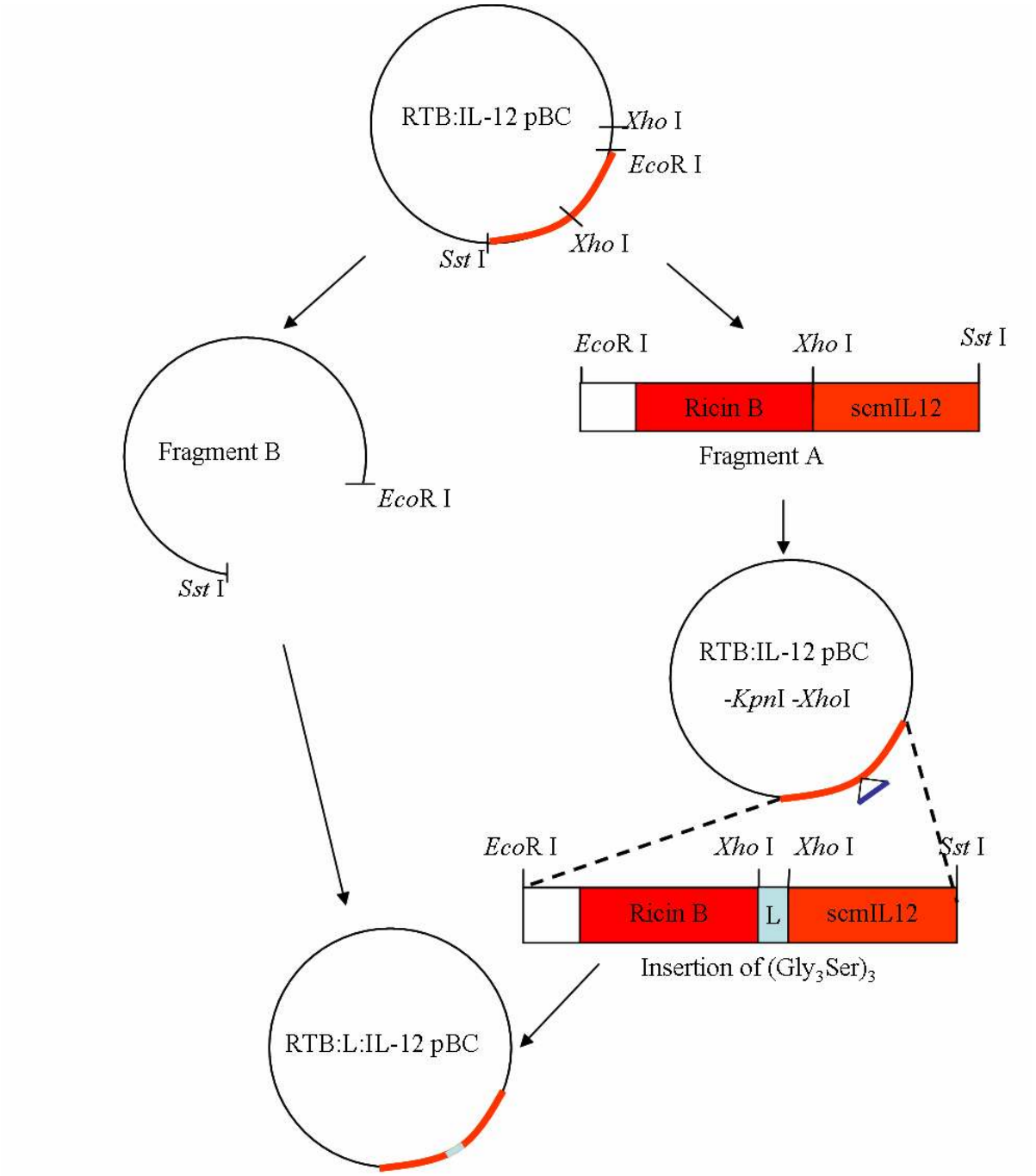


Fig.Ⅳ.3 Generation of RTB:L:IL-12 construct. RTB:IL-12 in pBC construct was digested by *Eco*RI and *Sst*I so that it was divided in two fragments, A and B. Fragment A containing *TEV:pat:RTB:IL-12* was inserted into a modified pBC vector (without *Xho*I and *Kpn*I restriction sites) permitting introduction of an oligonucleotide encoding $(Gly_3Ser)_3$ into the *Xho*I site the between RTB and mIL-12. Fragment A with the linker was then ligated back to fragment B which contains *35S* promoter.

et al., 1997) by PCR with primer 5'- GGTACCATGGGTCCTCAGAAGCTAA and primer 5'-GAGCTCGGCGGAACTCAGATAGCC. Sequences encoding RTB were amplified from plasmid R6-2 (Medina-Bolivar et al., 2003) using primers 5'-GAGCTCGCTGATGTTTGTATGGA and 5'-GTCGACTCAAAATAATGGTAACCATA which added a *Sac*I site to the 5' end and an in-frame stop codon and *Sal*I site to the 3' end. DNA fragments including the double enhanced *35S:TEV* promoter digested from plasmid R6-2 (Medina-Bolivar et al., 2003), *IL-12* and *RTB* with stop codon were assembled into pBC by multiple digestions and ligations to yield the construct *IL-12:RTB* (Fig.Ⅳ.2).

Ⅳ.3.4 Plant transformation

All constructs were introduced into the binary vector pBIB-Kan (Becker, 1990) and transformed into tobacco (*Nicotiana tabacum*) cv. Xanthi using *Agrobacterium*-mediated transformation as described previously (Chapter 2, Ⅱ.3.2).

Ⅳ.3.5 IL-12 ELISA

The expression levels of IL-12:RTB and RTB:IL-12 were determined by IL-12 ELISA as described previously (Chapter 2, Ⅱ.3.3). It should be noted that this ELISA uses the p70 monoclonal antibody as the capture antibody. This antibody only binds to IL-12 in the proper conformational structure.

Ⅳ.3.6 Asialofetuin-binding assay

Crude extracts from transgenic plants expressing the various RTB fusions were investigated for RTB carbohydrate-binding activity using an asialofetuin-binding assay (Dawson et al., 1995). Microtiter plates were coated with 300 μg/ml of asialofetuin (calf fetuin digested with sialidase, Sigma) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), 100 μl/well, overnight at room temperature. After being blocked with 1% BSA in PBS for 1 hour, 96-well plates were incubated with samples for 1 hour. For detection of bound RTB, plates were subsequently incubated with anti-ricin rabbit IgG (1:3000, Sigma, St. Louis, MO), goat anti-rabbit IgG conjugated with alkaline phosphatase (1:3000, Bio-Rad, Hercules, CA) and phosphatase substrate (Pierce) and absorbance was read at 415nm.

Ⅳ.3.7 SDS-PAGE and western blotting analysis

Plant tissue was ground in two volumes of PBS containing 20 mM galactose. Crude extracts (20 μg of total soluble protein per sample) or purified samples were boiled with 1×SDS gel loading

buffer for 5 min and resolved by 10% SDS-PAGE (Invitrogen, Carlsbad, CA). Proteins were subsequently stained using a silver stain kit (GBiosciences, St. Louis, MO) or Coomassie blue, or blotted onto nitrocellulose membranes (Bio-Rad). For polyclonal anti-mIL-12 western blotting, membranes were subsequently blocked with 1% BSA in PBST (PBS with 0.1% of Tween 20) for 1 hour at room temperature, incubated with goat anti-mIL-12 neutralizing antibody (1:10,000, R&D Systems, Minneapolis, MN) in 1% BSA/PBST for 1 hour and rabbit anti-goat whole IgG alkaline phosphatase conjugate (1:10,000, Sigma) in 1% BSA/PBST for 45 min. Detection was finished by using CDP-Star (Roche, Indianapolis, IN) and Nitroblock Enhancer II (TROPIX, Bedford, MA) following manufacturers' protocols.

The process of anti-RTB western blotting was almost the same as for anti-mIL-12 western blotting except rabbit anti-RTB antibody (1:5000, Reidy and Cramer, unpublished) was applied to probe the membranes followed with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:5000, Bio-Rad).

Ⅳ.3.8 Lactose affinity chromatography

About 2 ml of crude plant extracts were batch-purified with 100µl lactose-conjugated agarose (CAT # CG004-5, EY Laboratories Inc., San Mateo, CA) overnight at 4° C with gentle rocking. The mixtures were then loaded onto empty columns and centrifuged at 13,000×g. After washing once with 100 µl of PBS, proteins that bound to lactose resin were eluted by running 100 µl of PBS containing 0.25 M galactose each time through the column twice (Reidy and Cramer, unpublished procedure).

Ⅳ.3. 9 Southern hybridization analysis

Plant genomic DNA was extracted from leaf tissue by using Nucleon PHYTOPURE DNA extraction kit (Amersham) following manufacturer's protocols. Genomic DNA was digested by either *Hin*dIII or *Sac*I, size-separated by 0.8% agrose gel electrophoresis and electroblotted onto hydrogen cellulose membrane (Amersham, Buckinghamshire, UK). In hybridization solution (260mM sodium phosphate, 7% SDS, 1 mM EDTA, and 1% BSA, pH 7.2), membranes were probed using ~800bp fragments amplified from mIL-12 pBC plasmid and labeled with dCTP-^{32}P (PerkinElmer, Boston, MA) by using Prime-it RmT Random Primer Labeling Kit (Stratagene). After 2 days incubation at 65 °C, the membrane was washed in hybridization wash solution (20 mM sodium phosphate, 1% SDS, 1 mM EDTA, 30 mM NaCl, pH 7.2) several times and exposed on film (XMR, CAT# 1651496) for two days at -80 °C.

Ⅳ.3.10 Development of hairy roots

Hairy roots were developed from selected transgenic tobacco lines expressing high levels of RTB-IL12 fusions as described in previous chapters (Chapter 2, II.3.2). Liquid cultures were initiated with ~20 root tips (1 cm) in a 250 ml flask containing B5 media (50 ml) and maintained under continuous light with shaking (90 rpm). About 1-week old hairy root cultures (50 ml media/250 ml flask) were transferred to PYREX® 2800 mL Fernbach-Style Culture Flasks (Item #4420-2XL, Corning) containing 0.5 L media and cultured for an additional 2 weeks. Tissues were then harvested and stored at -80 °C.

Ⅳ.3.11 Purification of plant-derived mIL-12:RTB

Hairy roots tissue was ground to a powder in liquid nitrogen and then homogenized in 2 volumes of grinding buffer (100 mM phosphate buffer, 20 mM galactose, pH 7.6). Prior to chromatography, supernatants were diluted two-fold by adding an equal volume of deionized H_2O. The resulting mixture was filtered through a 0.45 μm membrane (Pall, Ann Arbor, MI) and subjected to cation exchange chromatography (Uno S column, Bio-Rad). Plant-derived mIL-12:RTB (IL-12:RTB) was eluted by a salt gradient from 0-1 M NaCl in phosphate buffer. Fractions containing mIL-12 were pooled and applied to a lactose affinity column (CAT# CG004-5, EY Laboratories, self-packed). mIL-12:RTB was then eluted using 0.25 M galactose in PBS. Fractions were examined by SDS-PAGE and visualized by silver staining. Eluates containing mIL-12:RTB were dialyzed against PBS, concentrated (Centricon YM-30, Millipore), quantified by mIL-12 p70 ELISA (R&D Systems, Minneapolis, MN) and stored at 4 °C. Visualization of silver-stained gels was used to estimate purity of IL-12:RTB.

Ⅳ.3.12 IL-12 biological activity assay

IL-12 activity was determined by induction of IFN-γ in primary splenocytes from C57BL/6 mice and by stimulation of splenocyte proliferation as described in Chapter 2 (II.3.8; II.3.9). The results are given as mean±1 standard deviation.

Ⅳ.3.13 Culture of HT-29 cell monolayer

HT-29 cells (ATCC # HTB-38) were seeded onto cell culture inserts (CAT# PIHP01250, Millipore, Bedford, MA) at $7.5\times10^4/cm^2$ and cultured in cell culture media (McCoy's 5a supplemented with 5 mM HEPES, 2 mM glutamine, 10% heat inactivated FBS, 100 U penicillin and 100 μg streptomycin; Gibco, Grand Island, NY) for about 3-4 weeks, till cell monolayers formed tight

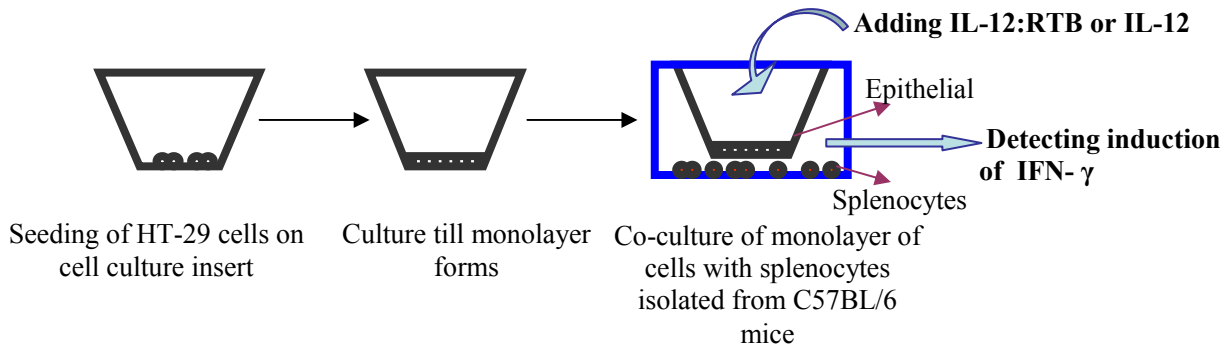


Fig.Ⅳ. 4 Schematic of HT-29 cells and splenocytes co-culture procedure for an *in vitro* MALT model for RTB transport assays.

conjunctions (Fig.Ⅳ.4). To test the integrity of cell monolayer, the inserts were washed 3 times with 1% DMSO (Sigma) in Hank's balanced salt solution (HBSS, Gibco) and 200 μl of 60 μM of Lucifer yellow (Sigma) was added to the inserts. The inserts were placed in a 24-well plate (Greiner Bio-One, Monroe, NC) with 300 μl of 1% DMSO/HBSS in each well and cultured for 1 hour at 37 °C/5% CO2. The concentration of Lucifer yellow in the bottom was measured by using a fluorescent plate reader (excitation 485 nm/ emission 520 nm). The cell monolayer was considered to be tightly conjunct when the concentration of Lucifer yellow was below 5 μM in the lower chamber of the well. Each insert was examined by this integrity test before it was used for the transport assay.

Ⅳ.3.14 IL-12:RTB activity test in an *in vitro* MALT cell culture model

In order to assess the mucosal delivery potential of IL-12:RTB, plant-derived mIL-12 or mIL-12:RTB was added to the cell monolayer insert and the ability of IL-12 to stimulate IFN-γ production in splenocytes placed below the insert was determined (Fig.Ⅳ.4). To initiate RTB-mediated transport assays, the HT-29 monolayer inserts were washed three times with cold Hank's balanced salt solution (HBSS) and then treated with 0.1% BSA/HBSS with 140 ng of plant-derived mIL-12:RTB or mIL-12 and incubated at 4 °C for 30 min. These inserts were then washed 3 times with warm cell culture media and incubated with 400 μl of cell culture media (as described in Ⅳ.3.13) in each well. These inserts were then placed in a 24-well cell culture plates (Greiner Bio-One) with 600 μl of cell culture media in each well and incubated at 37 °C for 1 hour to permit endocytosis of bound RTB.

To provide mIL-12 responsive cells, mouse splenocytes were isolated from 8-20 weeks old C57BL/6 mice (Jackson Laboratory) and placed in 24-well culture plates at 7.5×10^5 cell/well in cell culture media (RPMI1640, 10% heat inactivated FBS, 5 mM HEPES, 2 mM glutamine, 100 U penicillin and 100 μg streptomycin) with 10 ng/ml of rhIL-2 (R&D Systems, Minneapolis, MN). The animal care was the same as described previously in Chapter 2 and Chapter 3. The inserts that have been treated with IL-12:RTB or plant-derived IL-12 were put into these splenocytes wells and co-cultured overnight. The inserts were removed and the remaining splenocytes were cultured for an additional day. The supernatants from the 24-well plate were then collected and tested for IFN-γ by ELISA kit (R&D Systems, Minneapolis, MN). The results are given as mean±1 standard deviation.

For RTB neutralization, 140 ng of mIL-12:RTB was mixed with RTB antibody (Reidy and Cramer, unpublished; 1:1000) before it was added to the inserts for cold treatment. The rest procedure was the same as described above. The results are given as mean±1 standard deviation.

Ⅳ.4 Results

Ⅳ.4.1 Expression of RTB and mIL-12 fusions in transgenic plants

For expression of RTB and mIL-12 fusions in transgenic plants, constructs that fused RTB to either the N-terminus (*RTB:IL-12*) or C-terminus (*IL-12:RTB*) of IL-12 were generated by utilizing *RTB* from plasmid R6-2 (Medina-Bolivar et al., 2003) and a single chain form of mIL-12 (Lieske et al., 1997). Constructs utilized a strong constitutive promoter *de35S* (the dual-enhanced 35S promoter, Lam et al., 1989), the tobacco etch virus (*TEV*) translational enhancer (Carrington et al., 1990), and sequences encoding the signal peptide provided either by the patatin signal peptide (pat, Iturriaga et al., 1989) upstream of *RTB* (Fig.Ⅳ.2), or the mIL-12 sequences (p40, endogenous) depending on orientation. These two constructs were then inserted into pBIB-Kan (Becker, 1990) for transformation of the plant cell via *Agrobacterium tumefaciens*-mediated transformation. Because the site of transgene insertion has significant impacts on expression in plants, we generated and screened 30-40 independent transformants for each construct. Crude leaf extracts of these transgenic plants were then screened by IL-12 conformational ELISAs which is correlated to the bioactivity of IL-12 (Chapter 2) and by asialofetuin-binding assays to assess RTB lectin activity. Asialofetuin contains 12 terminal galactose residues per molecules and exhibits high affinity for RTB, the carbohydrate-binding subunit of ricin (Dawson et al., 1995). As shown in Table Ⅳ.1, among *RTB:IL-12* transgenic plants, less than a quarter of them showed detectable levels of mIL-12 in crude extracts, and only one of those exhibited low level of functional RTB activity. In contrast, the majority of *IL-12:RTB* transgenic plants expressed functional mIL-12 and RTB, and the levels of mIL-12 were correlated to the levels of RTB in most plants (data not shown). The best expressers of both constructs were utilized for further characterization.

Table Ⅳ.1: Screening of plants transformed by RTB:mIL-12 fusion constructs.

Constructs	Number of plants screened	No. of responsive plant lines	
		IL-12 ELISA (+)*	Asialofetuin-binding assay (+)*
RTB:IL-12	38	9	1
IL-12:RTB	34	21	22

*When the absorbance of screened plant is greater than that of non-transgenic control, it is positive.

Crude leaf extracts from transgenic plants expressing fusion proteins were characterized by anti-mIL-12 western blotting analysis. *RTB:IL-12* plants showed a protein band of approximately ~110 kDa that cross-reacted with anti-mIL-12 antibody (Fig.Ⅳ.5, lane 4), suggesting the production of a full

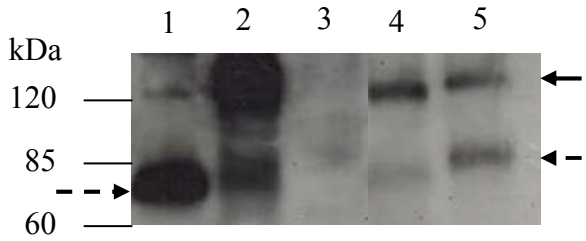


Fig.Ⅳ. 5 Western blotting analysis of RTB-mIL-12 fusion from transgenic plants. Proteins from crude extract (20 μg total soluble protein, lane 2-5) of transgenic plants and animal cell-derived IL-12 (acdIL-12, 50 ng, lane 1) were resolved on a 10% SDS-PAGE gel under non-reducing conditions and transferred to nitrocellulose membrane. The membrane was probed with polyclonal mIL-12 antibody. The solid arrow points out the full length of fusion protein (around 110-120 kDa). The dashed arrow on the left shows mIL-12 p70 and the dashed arrow on the right indicates the breakdown products of the fusion proteins (around 70 kDa). Lane 1: animal cell derived IL-12; lane 2: extracts from plants transformed by *IL-12:RTB* construct; lane 3: extracts from plants expressing RTB:GFP fusion (non-IL-12 control; Medinar-Bolivar et al., 2003); lane 4: extracts from plants transformed by *RTB:IL-12*; lane 5: extracts from plants transformed by *RTB:L:IL-12* construct. Expression levels in plants producing IL-12:RTB were significantly higher than those producing RTB:IL-12 or RTB:L:IL-12. Film was over-exposed for lanes 1 and 2 in order to visualize lanes 4 and 5.

length RTB:mIL-12 in transgenic plants. In addition to this ~110kDa band, there are other bands with faster mobility on SDS-PAGE that also cross-reacted with mIL-12 antibody. These bands are not present in non-mIL-12 transgenic extracts (Fig.Ⅳ.5, lane 3) or non-transgenic controls (data not shown), suggesting they may represent breakdown products of full length RTB:mIL-12 (RTB:IL-12). The breakdown of RTB and mIL-12 in tobacco extracts has already been noted (Medina-Bolivar et al., 2003; Chapter 2). *IL-12:RTB* transgenic plants produced a protein band with slightly higher molecular weight (~120kDa) than those from *RTB:IL-12* plants (Fig.Ⅳ.5, lanes 2 and 4), probably due to different post-translational modification or conformation. Similar to those from *RTB:IL-12* plants, extracts from *IL-12: RTB* plants also demonstrated breakdown products of mIL-12:RTB (IL-12:RTB), which cross-reacted with mIL-12 antibody (Fig.Ⅳ.5, lane 2).

Ⅳ.4.2 IL-12:RTB, but not RTB:IL-12, retains full lectin activity

It has been shown that both *IL-12:RTB* and *RTB:IL-12* transgenic plants produce full length fusion proteins. However, initial screening with the asialofetuin-binding assay suggested that the RTB:IL-12 arrangement resulted in products with reduced carbohydrate-binding activity. To explore this further, we utilized lactose-affinity chromatography, which is routinely used for affinity purification of RTB. As shown in Fig.Ⅳ.6, the full length IL-12:RTB binds to lactose affinity column and can be eluted by high concentration of galactose (Fig.Ⅳ.6, lane 1 and 2), suggesting that RTB fused to the carboxyl-terminus of IL-12 retains lectin activity. In contract, the full length RTB:IL-12 was not recovered in the galactose eluate from lactose columns when the leaf extracts of RTB:IL-12 transgenic plants were applied to the column (Fig.Ⅳ.6, lane 3 and 4). This suggests that IL-12 when fused to the carboxyl-terminus of RTB interferes with RTB lectin activity, perhaps due to steric hindrance and/or masking of active domains of RTB.

In an effort to address whether the lack of lectin activity in RTB:IL-12 fusion is caused by steric hindrance, a polypeptide linker $(Gly_3Ser)_3$ was inserted in between RTB and mIL-12 in the *RTB:IL-12* construct. The resulting construct, *RTB:L:IL-12*, was utilized for plant transformation as described above. This 12-amino acid glycine-rich linker is very flexible in structure (Robinson et al., 1998) and may provide additional spacing to enable RTB and IL-12 to maintain functional three-dimensional structure. *RTB:L:IL-12* plants produced a protein band slightly higher in molecular weight than the one from *RTB:IL-12* plants (Fig.Ⅳ.5, lane 5), which is consistent with the presence of the polypeptide linker. The conformational IL-12 ELISA suggests that RTB:L:IL-12 maintains IL-12 bioactivity (data not shown). The full length RTB:L:IL-12 also binds to the lactose column (Fig.Ⅳ.6,

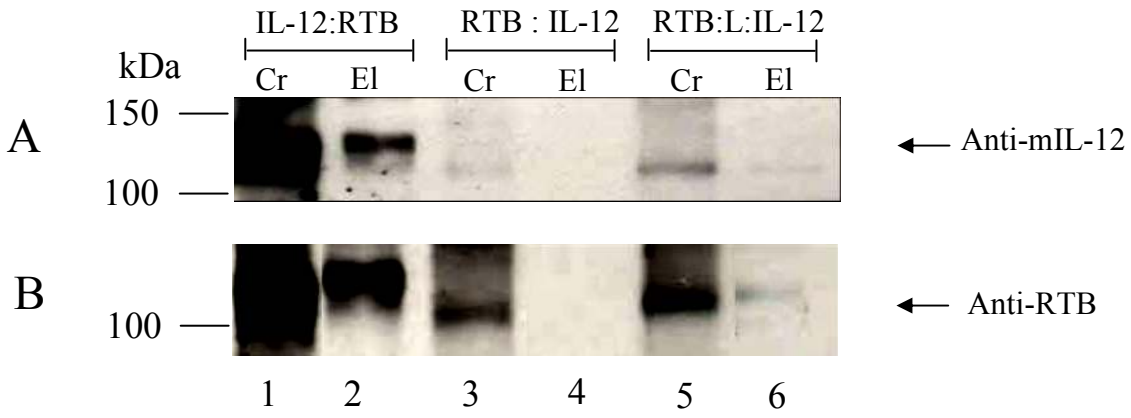


Fig.Ⅳ. 6 Comparison of RTB-IL-12 fusions recovered by lactose affinity chromatography. Crude leaf extracts (Cr, 20 μg of total soluble protein, lanes 1, 3, 5) from transgenic plants were batched with lactose resin overnight at 4°C. Proteins bound to lactose were eluted by 0.25 M galactose in PBS (El, 20 μl, lanes 2, 4, 6). Samples were resolved on a 10% SDS-PAGE gel under non-reducing conditions and transferred to nitrocellulose membrane. The membrane was probed with polyclonal mIL-12 antibody (A) or RTB antibody (B). Lane 1 and 2: from IL-12:RTB expressing plants; lane 3 and 4: from RTB:IL-12 expressing plants; lane 5 and 6: from RTB:L:IL-12 expressing plants.

lanes 5 and 6), suggesting that the presence of polypeptide linker aids the fusion partners to retain their respective bioactivities.

Ⅳ.4.3 Characterization of plant-derived IL-12:RTB

Although RTB:L:IL-12 maintains IL-12 conformation and RTB lectin activity, its expression level in transgenic plants, like that of RTB:IL-12, is much lower than that of IL-12:RTB plants (Fig.Ⅳ.5, lane 2 and 5). As a result, IL-12:RTB plants were chosen for further characterization and *in vitro* biological activity assays. The top two expressers, IL-12:RTB plants IR6-11 and IR6-17 were analyzed by Southern hybridization to confirm transgene integration and copy number. Our result showed that each transgenic plant has 3 copies of transgene integrated (data not shown). Hairy roots, a rapid biomass accumulating culture system, were developed from these top two expressers via *Agrobacterium rhizogenes* (ATCC#15834)-mediated transformation (Medina-Bolivar and Cramer, 2004). Approximately 50 g of hairy roots can be recovered from one liter of media after 21-day culture.

A number of grinding buffers were tested for maximum extraction of the transgenic product from hairy roots. A simple buffer, 20 mM galactose in 100 mM sodium phosphate buffer (pH 7.6), recovered the most transgenic products from the plant tissue (data not shown). Galactose was introduced into the grinding buffer based on the rationale that galactose can compete for the galactose-binding domains on RTB, thus enhancing IL-12:RTB release from the glycan-rich plant tissue (Reidy and Cramer, unpublished).

In order to effectively assess RTB-mediated uptake and delivery of functional IL-12, it was necessary to purify the product. It has previously been shown that mIL-12 can be purified from plant extracts by cation-exchange chromatography (Chapter 2) and RTB can be affinity-purified leveraging its lactose binding property (Fig.Ⅳ.6; Reidy and Cramer, unpublished). Therefore, a two-step protein purification scheme combining cation-exchange and lactose affinity chromatography was developed for plant-derived IL-12:RTB purification. This purification scheme recovers about 30% of IL-12:RTB from the crude extraction and typically yields about 1 μg of purified protein per gram of hairy root fresh weight. All quantitation of purified IL-12:RTB was based on IL-12 p70 conformational ELISA.

The purified plant-derived IL-12:RTB showed above 90% purity on silver-stained SDS-PAGE gel under non-reducing condition (Fig.Ⅳ.7 A). However, under reducing condition, beside the full length protein bands, there were also many smaller size bands ranging from ~30 kDa to ~90 kDa

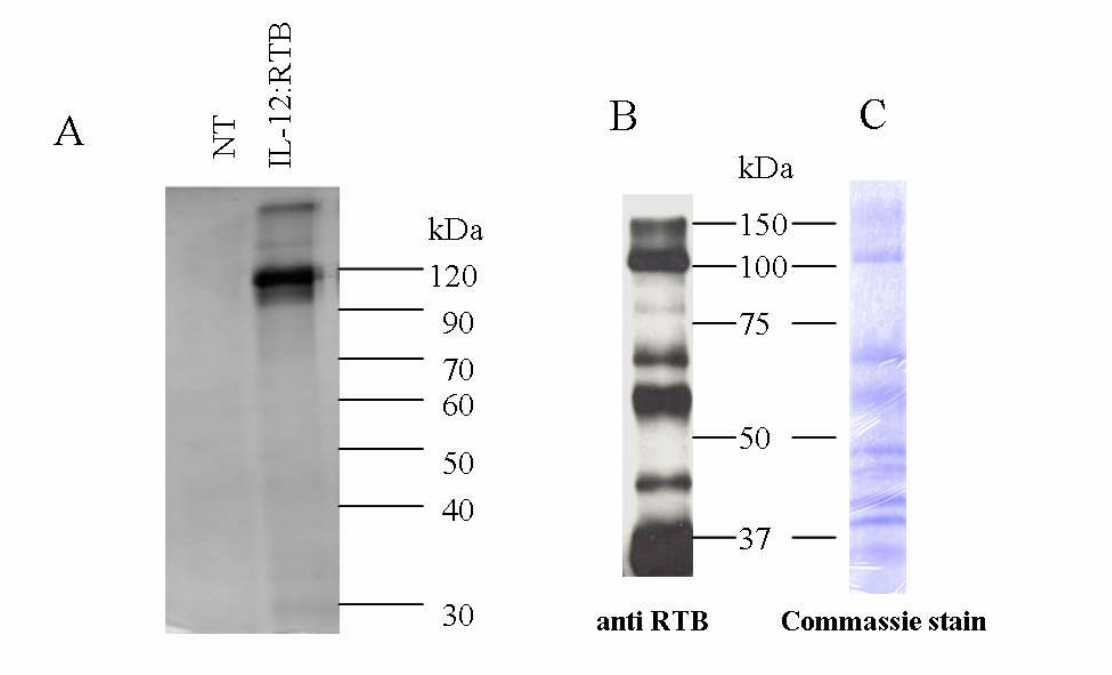


Fig.Ⅳ. 7 SDS-PAGE and western blotting analysis of purified IL-12:RTB. (A) About 100 ng of purified IL-12:RTB (IL-12:RTB) and equivalent fractions from non-transgenic control (NT) were resolved by 10% SDS-PAGE under non-reducing condition and silver stained to visualize proteins. (B) Purified IL-12:RTB was resolved by 10% SDS-PAGE gel under reducing condition and proteins were then either transferred to membrane for anti-RTB western blotting analysis (~100 ng, B), or stained by Coomassie stain (~4 μg, C).

(Fig.Ⅳ.7 B). At least four of these breakdown bands cross-reacted with anti-RTB antibody (Fig.Ⅳ.7 C). These results suggest that some peptide bonds in plant-derived IL-12:RTB probably have been cleaved by plant proteases. However, these breakdown products still assemble together likely through disulfide bond interactions and both fusion partners maintain their respective biological functions. The potential proteolytic sites may be identified by sequencing the N-terminus of these breakdown protein bands.

Ⅳ.4.4 Plant-derived IL-12:RTB exhibits IL-12 biological activity *in vitro*

To confirm plant-derived IL-12:RTB maintains full IL-12 bioactivity, this purified recombinant fusion protein was studied for *in vitro* bioactivity in cultured splenocytes isolated from C57BL/6 mice. The results showed plant-derived IL-12:RTB demonstrated a dose-dependent activity in inducing the secretion of IFN-γ from splenocytes and stimulation of the proliferation of PHA pre-activated splenocytes, similar to the bioactivity of plant-derived mIL-12 as shown in Chapter 2 (Fig.Ⅳ.8 A and C). These activities were blocked by pre-incubating IL-12:RTB with mIL-12 neutralizing antibody, suggesting they represent mIL-12 specific activities (Fig.Ⅳ.8 B and D). From these assays, we also found that RTB (100 ng/ml; Vector laboratory, Inc.) alone did not stimulate IFN-γ secretion (data not shown). RTB neutralization with anti-RTB antibody did not block IL-12 activity (data not shown).

Ⅳ.4.5 RTB facilitate the uptake of its fusion partner mIL-12

Our results have shown that plant-derived IL-12:RTB exhibited IL-12 bioactivity and functional RTB activity. The purpose of producing IL-12:RTB fusion was to test whether RTB can function as a molecular carrier to facilitate the mucosal delivery of IL-12. Therefore, an *in vitro* transport assay model was modified to simulate the epithelial surface found in mucosal-associated lymphoid tissue (MALT; Fig.Ⅳ.1). The monolayer of HT-29 cells (a human intestinal epithelial cell line) has been routinely utilized as an *in vitro* model of the intestinal epithelium to study drug transport and metabolism (Behrens et al., 2001; Blais et al., 1997; Thomson et al., 1997; Walter et al., 1996). We modified this intestinal epithelium model by adding splenocytes as immune responsive cells to the basolateral side of monolayer (Fig.Ⅳ.4). RTB uptake experiment showed that RTB binds to the surface of cultured HT-29 cells and enters the cell through endocytosis (Liu, Reidy and Cramer, unpublished). We hypothesized that RTB may mediate epithelial cell uptake of IL-12 and delivery of the fusion product across the HT-29 monolayer to stimulate the IL-12 responsive splenocytes in the well below to produce IFN-γ.

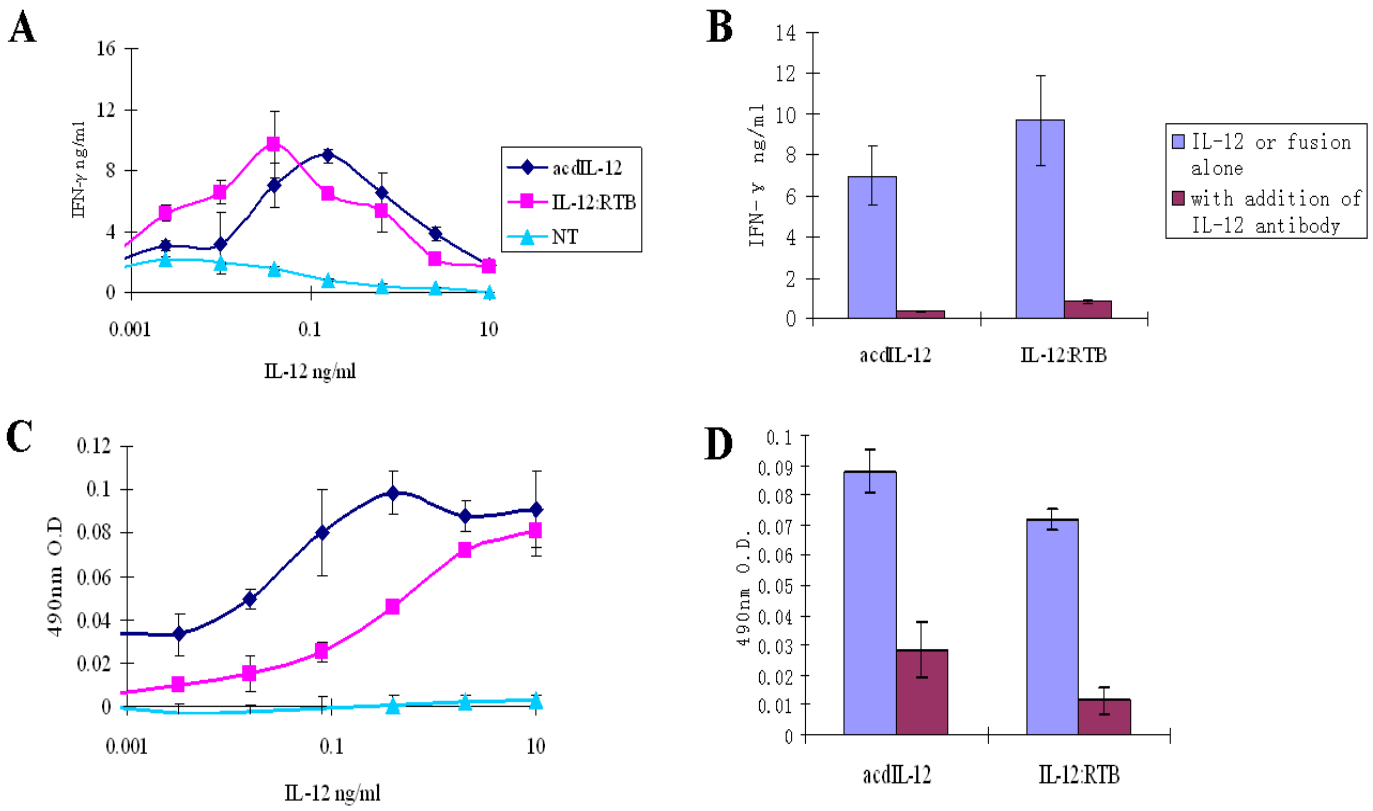


Fig.Ⅳ. 8 IL-12 bioactivity assay in mouse splenocytes. (A) Splenocytes from C57BL/6 mice were cultured for 48 hours, with 10 ng/ml rhIL-2 and the indicated amounts of animal cell-derived mIL-12 (acdIL-12), IL-12:RTB purified from transgenic hairy roots (IL-12:RTB), or equivalent fractions from non-transgenic hairy roots controls (NT). Supernatants were assayed for IFN-γ concentration by ELISA. (B) Induction of IFN-γ in the presence or absence of IL-12 neutralizing antibody. IL-12 from different sources was incubated with or without IL-12 neutralizing antibody for 2 hours prior to addition to mouse splenocytes cultures. The procedure of cell culture was the same as in (A). Data from 0.04 ng/ml of IL-12 is shown. (C) Standard colorimetric cell proliferation assays were performed on splenocytes as described in Material and Methods. PHA-preactivated splenocytes were cultured for 48 hours with 10 ng/ml rhIL-2 and the indicated amounts of acdIL-12, IL-12:RTB, or NT control. (D) Comparison of cell proliferation in splenocytes treated with IL-12 in the presence (red bars) or absence (blue bars) of neutralizing antibodies. The procedure of cell culture and cell proliferation assays was the same as in (C). Data from 2 ng/ml of IL-12 is shown. Data points represent the mean ±1 standard deviation of duplicate wells. These data are representative of three separate experiments.

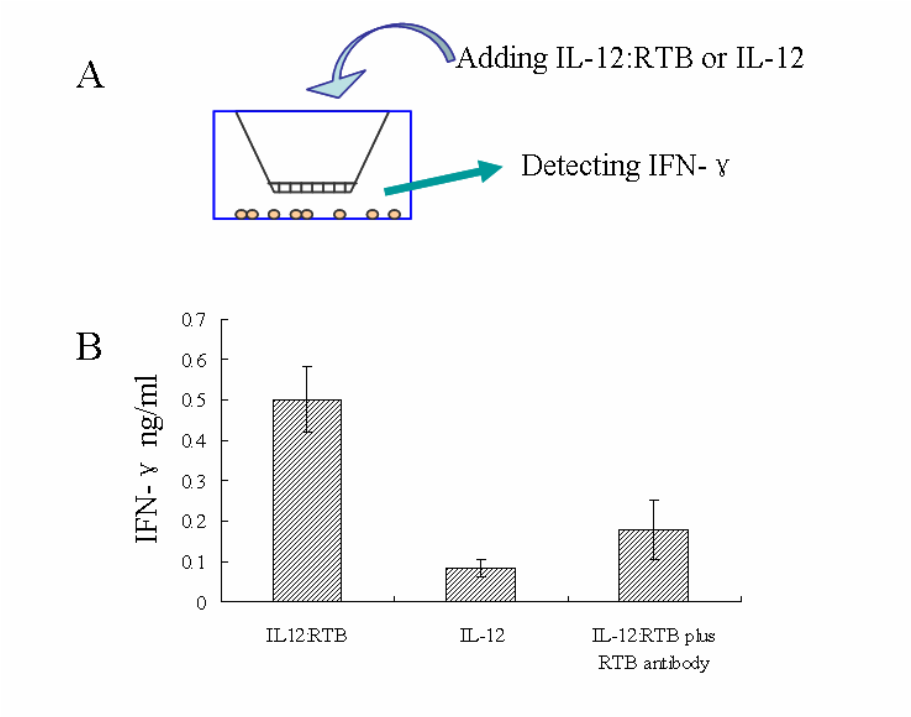


Fig.Ⅳ. 9 Test of purified IL-12:RTB in a MALT *in vitro* model. (A) Schematic of testing IL-12:RTB in a MALT *in vitro* model. (B) About 140 ng of purified IL-12:RTB or IL-12 from transgenic plants was added to inserts containing a HT-29 monolayer and incubated at 4 °C for 30 min and to allow RTB to bind to the cell surface. After being washed 3 times, the monolayer of cells was then co-cultured with splenocytes from C57BL/6 mice as described in Material and Methods. Supernatants of splenocytes were collected for IFN-γ ELISAs. For RTB neutralization, RTB antibody (1:1000) was added to 140 ng of IL-12:RTB prior to incubation with monolayer of cells at 4 °C. The rest was the same as described above. Data points represent the mean ±1 standard deviation of triplicate wells. The data are representative of two separate experiments.

The MALT model was characterized before it was used in plant-derived IL-12:RTB investigation. These HT-29 monolayer inserts were examined for integrity by Lucifer yellow rejection before they were used for *in vitro* MALT model. The rejection rate of each insert was above 80%. In each experiment, plant-derived mIL-12 was used as the control for "leakage", i.e. splenocyte stimulation caused by limited amount of IL-12 that moves between cells of the monolayer. Because we found that purified plant-derived RTB (Reidy and Cramer, unpublished) did not trigger splenocytes to produce IFN-γ (data not shown), RTB control was not included in the experiments.

Plant-derived IL-12:RTB and controls were applied to the apical side of the cell monolayer to investigate whether IL-12 can be transported to the basolateral side and activate splenocytes (Fig.Ⅳ.9 A). After culturing for 2 days, supernatants of splenocytes were collected for IFN-γ ELISA. Our results showed that IL-12:RTB stimulated the splenocytes to secret IFN-γ (Fig.Ⅳ.9 B), suggesting that RTB facilitates the delivery of its covalently linked fusion partner IL-12 from the apical to the basolateral surface of monolayer where IL-12 stimulated responsive cells in the splenocyte culture. In contrast, much less of IFN-γ was produced from splenocytes when co-culturing with HT-29 cell monolayer that has been treated with equivalent amount of plant-derived IL-12 (Fig.Ⅳ.9 B).

IL-12:RTB, co-incubated with anti-RTB polyclonal antibody also showed reduced potential to stimulate IFN-γ production in the splenocytes below suggesting the RTB activity was vital in mediating delivery of IL-12 across the HT-29 cell monolayer. Our results strongly support that RTB functions as a molecular carrier and facilitates the delivery of IL-12 to the mucosal epithelium to immune responsive cells in this model.

Ⅳ.5 Discussion

IL-12 shows great promise as an anti-tumor therapeutic and a viral and cancer vaccine adjuvant (reviewed by Colombo and Trinchieri, 2002). The clinical application of IL-12 has been hindered by its toxicity associated with systemic administration (Leonard et al., 1997). However, it has been reported that localized delivery of IL-12 is effective and less toxic (reviewed by Salem et al., 2006). We have explored the potential that RTB, the non-toxic glycan-binding subunit of ricin, may function as a molecular carrier to facilitate the mucosal delivery of IL-12. Previous studies have shown that RTB and mIL-12 can be successfully produced in transgenic plants, respectively (Chapter 2; Reidy and Cramer, unpublished; Medinar-Bolivar et al., 2003; Reed et al., 2005). In this chapter, mIL-12:RTB (IL-12:RTB) fusions were produced in transgenic plants and the bioactivity of both fusion partners was demonstrated. An effective purification scheme was developed for plant-derived IL-12:RTB yielding

approximately 1 μg of purified protein per gram of fresh weight of hairy roots. The purified fusion protein stimulated production of IFN-γ in mouse splenocytes (mIL-12 bioactivity) and showed high binding affinity to target glycoproteins (RTB bioactivity). An *in vitro* model of MALT was utilized to demonstrate that RTB facilitates the delivery of mIL-12 through a cultured cell monolayer to immune responsive cells.

It is generally accepted that orientation of fusion partners may be critical to produce functional fusion proteins (Wittenmayer et al., 2000). Our results demonstrate that RTB fused to the amino-terminus of mIL-12 did not show full lectin activity. The insertion of a polypeptide glycine-serine linker $(Gly_3Ser)_3$ between RTB and mIL-12 resulted in an RTB-active lectin product that bound to lactose columns (Fig.Ⅳ.6). The glycine-serine linker is very flexible in structure (Robinson et al., 1998), which likely provided extra spacing for fusion partners to fold correctly without interference.

Plant-derived IL-12:RTB demonstrated full bioactivity and high expression levels in transgenic plants. Based on the gene construct that was used to generate transgenic plants (Fig.Ⅳ.2), the transgenic product should be a single-chain fusion protein. Under non-reducing condition, plant-derived IL-12:RTB showed a doublet at around 120 kDa on silver-stained SDS-PAGE gel (Fig.Ⅳ.7 A), probably due to different glycosylation forms of the fusion protein (see Chapter 2). However, multiple breakdown bands were observed on reducing SDS-PAGE gel (Fig.Ⅳ.7 B) with four bands cross-reacting with the RTB antibody (Fig.Ⅳ.7 C). This breakdown of single-chain protein in plant production system has been observed previously. The polypeptide linker $(Gly_4Ser)_3$ that combines the two subunits of mIL-12 together in this single-chain construct, has been shown to be susceptible to some plant proteases (Reidy and Cramer, unpublished; Chapter 2). Moreover, when RTB is fused to the carboxyl-terminus of its fusion partner, the amino-terminus of RTB has been shown to be susceptible to plant proteases (Reidy and Cramer, unpublished). Edman sequencing of these breakdown bands may help to identify the potential proteolytic sites in transgenic plants. Nevertheless, cleavage of these peptide bonds in plant-derived recombinant fusion proteins did not affect their respective bioactivities. Plant-derived mIL-12 and IL-12:RTB exhibited full bioactivities (Fig.Ⅳ.8; Chapter 2) and appear equivalent to animal-cell-derived mIL-12. For mucosal delivery of protein, if the protein does not bind to the mucosal tissue, most of it would be washed away by body fluids circulating around all the time. RTB binds to the glycan-rich mucosal tissue so that it may work as a molecular carrier and facilitate the delivery of the fusion partner to mucosal functional sites. Our results suggested that RTB facilitated the delivery of its fusion partner IL-12 through the epithelial

monolayer and as result stimulated the immune responsive cells positioned below the epithelial monolayer. Because RTB is involved in multiple transport pathways inside the cell (reviewed by Sandvig et al., 2000), it may help targeting its fusion partner to some specific organelles, and may assist the presentation of its fusion partner to different type of cells *in vivo* (Chapter 1, Fig.I.4). By further investigating IL-12:RTB in this *in vitro* MALT model, we may be able to disclose how this fusion protein is transported from the apical surface to the basolateral surface of monolayer of cells.

Monolayers of cultured epithelial cells have been studied as *in vitro* model for drug and nutrient transport and metabolism (Behrens et al., 2001; Blais et al., 1997; Thomson et al., 1997; Walter et al., 1996). We modified this model by adding immune cells to the basolateral side of monolayer so that it can simulate the structure of MALT. Nevertheless, this model is far away from a mature model for extensive research. Many questions remain to be addressed for this MALT model, like lectin binding efficacy and transport dynamics and kinetics. This model may also be optimized for investigation of molecules as molecular carriers for delivery to mucosal tissue in many ways, such as replacing HT-29 cell and mouse splenocytes with other types of cells that satisfy the research purpose.

Although our results showed that IL-12:RTB facilitated the transport of IL-12 through cell monolayer and stimulated the secretion of IFN-γ from splenocytes, it is still necessary to apply this fusion protein in an *in vivo* mouse model to investigate whether IL-12:RTB is more efficient in inhibiting tumor growth or stimulating Th1 immunity than IL-12 alone is. Based on our *in vitro* MALT model described in this chapter, we would predict that IL-12:RTB would stimulate antigen-specific Th1-biased immunity at significantly lower concentrations of IL-12 and result in reduced serum IFN-γ levels which have been linked to toxicity.

Ⅳ.6 References

Becker D. Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. 1990; 18: 203.

Behrens I, Stenberg P, Artursson P, Kissel T. Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. Pharm.Res. 2001; 18: 1138-1145.

Blais A, Aymard P, Lacour B. Paracellular calcium transport across Caco-2 and HT29 cell monolayers. Pflugers Arch. 1997; 434: 300-305.

Carrington JC, Freed DD. Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. J.Virol. 1990; 64: 1590-1597.

Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002; 13: 155-168.

Dawson RM, Paddle BM, Alderton MR. Characterization of the Asialofetuin microtitre plate-binding assay for evaluating inhibitors of ricin lectin activity. J.Appl.Toxicol. 1999; 19: 307-312.

Endo Y, Tsurugi K. RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J.Biol.Chem. 1987; 262: 8128-8130.

Frankel AE, Fu T, Burbage C et al. Lectin-deficient ricin toxin intoxicates cells bearing the D-mannose receptor. Carbohydr.Res. 1997; 300: 251-258.

Gutierrez-Ortega A, Avila-Moreno F, Saucedo-Arias LJ, Sanchez-Torres C, Gomez-Lim MA. Expression of a single-chain human interleukin-12 gene in transgenic tobacco plants and functional studies. Biotechnol.Bioeng. 2004; 85: 734-740.

Gutierrez-Ortega A, Sandoval-Montes C, Olivera-Flores TJ, Santos-Argumedo L, Gomez-Lim MA. Expression of functional interleukin-12 from mouse in transgenic tomato plants. Transgenic Res. 2005; 14: 877-885.

Huber VC, Arulanandam BP, Arnaboldi PM *et al* . Delivery of IL-12 intranasally leads to reduced IL-12-mediated toxicity. Int.Immunopharmacol. 2003; 3: 801-809.

Iturriaga G, Jefferson RA, Bevan MW. Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. Plant Cell 1989; 1: 381-390.

Kwon TH, Seo JE, Kim J, Lee JH, Jang YS, Yang MS. Expression and secretion of the heterodimeric protein interleukin-12 in plant cell suspension culture. Biotechnol.Bioeng. 2003; 81: 870-875.

Lam E, Benfey PN, Gilmartin PM, Fang RX, Chua NH. Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. Proc.Natl.Acad.Sci.U.S.A 1989; 86: 7890-7894.

Leonard JP, Sherman ML, Fisher GL et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood 1997; 90: 2541-2548.

Lieschke GJ, Rao PK, Gately MK, Mulligan RC. Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity *in vivo*. Nat.Biotechnol. 1997; 15: 35-40.

Magnusson S, Kjeken R, Berg T. Characterization of two distinct pathways of endocytosis of ricin by rat liver endothelial cells. Exp.Cell Res. 1993; 205: 118-125.

Medina-Bolivar F, Wright R, Funk V et al. A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine 2003; 21: 997-1005.

Medina-Bolivar F, Cramer C. Production of recombinant proteins by hairy roots cultured in plastic sleeve bioreactors. Methods Mol.Biol. 2004; 267: 351-363.

Mohanraj D, Ramakrishnan S. Cytotoxic effects of ricin without an interchain disulfide bond: genetic modification and chemical crosslinking studies. Biochim.Biophys.Acta 1995; 1243: 399-406.

Newton DL, Wales R, Richardson PT et al. Cell surface and intracellular functions for ricin galactose binding. J.Biol.Chem. 1992; 267: 11917-11922.

Reed DG, Nopo-Olazabal LH, Funk V et al. Expression of functional hexahistidine-tagged ricin B in tobacco. Plant Cell Rep. 2005; 24: 15-24.

Roberts LM, Smith DC. Ricin: the endoplasmic reticulum connection. Toxicon 2004; 44: 469-472.

Robinson CR, Sauer RT. Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. Proc.Natl.Acad.Sci.U.S.A 1998; 95: 5929-5934.

Salem ML, Kadima AN, Zhou Y et al. Paracrine release of IL-12 stimulates IFN-gamma production and dramatically enhances the antigen-specific T cell response after vaccination with a novel peptide-based cancer vaccine. J.Immunol. 2004; 172: 5159-5167.

Salem ML, Gillanders WE, Kadima AN et al. Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. J.Interferon Cytokine Res. 2006; 26: 593-608.

Sandvig K, van Deurs B. Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. EMBO J. 2000; 19: 5943-5950.

Smith DC, Gallimore A, Jones E et al. Exogenous peptides delivered by ricin require processing by signal peptidase for transporter associated with antigen processing-independent MHC class I-restricted presentation. J.Immunol. 2002; 169: 99-107.

Thomson AB, Doring K, Keelan M, Armstrong G. Nutrient uptake into undifferentiated and differentiated HT-29 cells in culture. Can.J.Physiol Pharmacol. 1997; 75: 351-356.

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994; 84: 4008-4027.

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat.Rev.Immunol. 2003; 3: 133-146.

van Deurs B, Hansen SH, Petersen OW, Melby EL, Sandvig K. Endocytosis, intracellular transport and transcytosis of the toxic protein ricin by a polarized epithelium. Eur.J.Cell Biol. 1990; 51: 96-109.

Walter E, Janich S, Roessler BJ, Hilfinger JM, Amidon GL. HT29-MTX/Caco-2 cocultures as an *in vitro* model for the intestinal epithelium: *in vitro-in vivo* correlation with permeability data from rats and humans. J.Pharm.Sci. 1996; 85: 1070-1076.

Wittenmayer N, Rothkegel M, Jockusch BM, Schluter K. Functional characterization of green fluorescent protein-profilin fusion proteins. Eur.J.Biochem. 2000; 267: 5247-5256.

Chapter V
Conclusions and Future Directions

IL-12 is a very important cytokine in mediating Th1 immunity. It induces the secretion of IFN-γ from preactivated T and NK cells, stimulates the cytotoxic activity of CTL cells and NK cells, and favors the differentiation of Th1 cells, linking innate immunity to adaptive immunity (Trinchieri, 1994; 2003). IL-12 has been investigated as an anti-tumor therapeutic and an adjuvant for cancer and viral vaccines, and exhibited great potential (reviewed by Colombo and Trinchieri, 2002; Salem et al., 2006). However, the clinical application of IL-12 was hindered by its toxicity associated with systemic administration of IL-12 (Leonard et al., 1997). Many research groups have demonstrated that localized delivery of IL-12 was as efficacious as systemic administration, with significantly reduced toxicity (Huber et al., 2003; Peng et al., 2001; Salem et al., 2004).

As IL-12 has a great potential as a therapeutic, there is significant demand for IL-12 for research purposes and clinical trials. The expression level of IL-12 in natural sources is too low to be purified for commercial applications. Currently, the commercially available IL-12 is from mammalian cell (CHO) or insect cell (*Sf*21) cultures. These culture systems are expensive to maintain and the yield of IL-12 is not very promising. There is currently no source of human IL-12 produced under cGMP condition to support clinical trials, or pharmaceutical commercialization.

My research has showed that transgenic plants successfully produced high amounts of bioactive murine IL-12. Hairy roots, a fast growing plant *in vitro* culture system, were developed from mIL-12 expressing transgenic plants. Mouse IL-12 represented more than 1% of total soluble protein in these cultures and about 150µg of purified mIL-12 can be obtained from one liter of hairy roots culture.

Purified plant-derived mIL-12 exhibited full biological activity in cultured splenocytes. It enhanced the secretion of IFN-γ from cultured mouse splenocytes and stimulated the proliferation of preactivated mouse splenocytes. Our investigation also showed that plant-derived IL-12 enhanced antigen-specific Th1 immunity in a mouse immunization model and was safe to be applied in mouse, suggesting transgenic plants can be utilized for IL-12 production. Another IL-12 mouse trial will be initiated soon to confirm plant-derived IL-12 has *in vivo* Th1 immuno-modulating activity and our plant-derived IL-12 has been provided to several investigators to test in a mouse anti-tumor model and a mouse influenza model.

Another goal of my research was to explore RTB as a molecular carrier for mucosal delivery of IL-12. In order to do that, transgenic plants expressing RTB-IL-12 fusions were generated and characterized. We found that RTB, when fused to the amino-terminus of IL-12, does not exhibit lectin activity due to steric hindrance. However, the opposite orientation, IL-12:RTB, showed not only RTB lectin activity but also full IL-12 biological activity *in vitro*. From IL-12:RTB expressing transgenic plants, hairy roots were developed for fast bioproduction of IL-12:RTB. Purified IL-12:RTB was

investigated in an *in vitro* MALT model. RTB facilitated the delivery of its fusion partner IL-12 through the cultured monolayer of epithelial cells.

My research demonstrated that transgenic plants are a feasible production system for functional IL-12. This plant-derived IL-12 may have broad applications in anti-tumor and anti-viral vaccines. Currently, plant-derived IL-12 is under investigation in a mouse influenza model for anti-viral activity by collaborating with a research group from Georgia. Furthermore, investigation of IL-12:RTB in *in vivo* mouse vaccination models via mucosal administration may determine whether IL-12:RTB would show greater efficacy (equivalent response at lower concentration) and less toxicity that those of IL-12 alone.

References:

Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002; 13: 155-168.

Huber VC, Arulanandam BP, Arnaboldi PM et al. Delivery of IL-12 intranasally leads to reduced IL-12-mediated toxicity. Int.Immunopharmacol. 2003; 3: 801-809.

Leonard JP, Sherman ML, Fisher GL et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. Blood 1997; 90: 2541-2548.

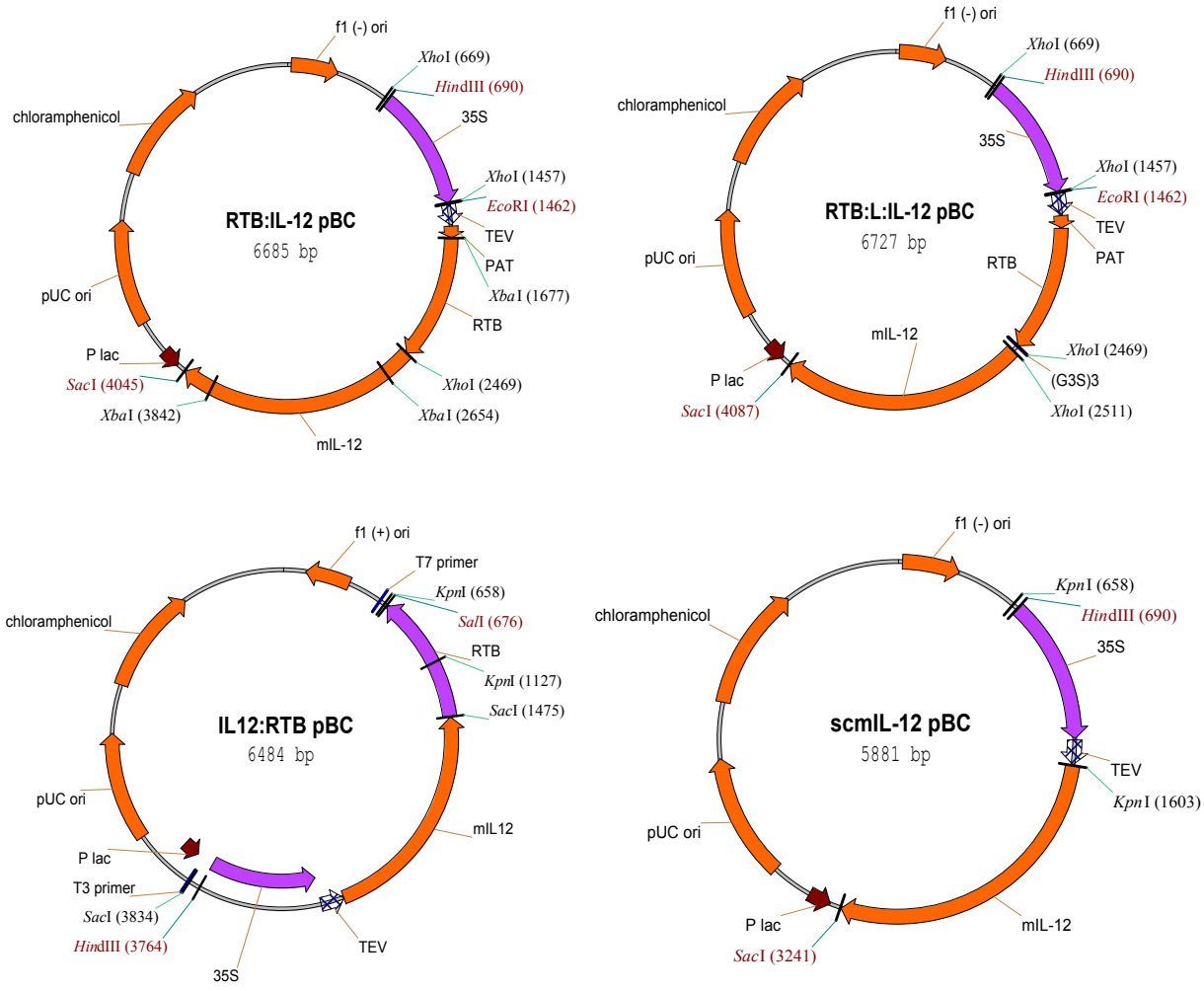
Peng LS, Penichet ML, Dela Cruz JS, Sampogna SL, Morrison SL. Mechanism of antitumor activity of a single-chain interleukin-12 IgG3 antibody fusion protein (mscIL-12.her2.IgG3). J.Interferon Cytokine Res. 2001; 21: 709-720.

Salem ML, Gillanders WE, Kadima AN et al. Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. J.Interferon Cytokine Res. 2006; 26: 593-608.

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994; 84: 4008-4027.

Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat.Rev.Immunol. 2003; 3: 133-146.

Appendix



A.1 Constructs in pBC (Stratagene). These maps were generated by *Vector* NTI software (Invitrogen). 35S: double enhanced 35S promoter; TEV: translational enhancer; PAT: DNA fragment encoding plant signal peptide; RTB: DNA fragment encoding ricin B subunit; mIL-12: DNA fragment that encoding murine IL-12 with or without endogenous signal peptide.

A.2. Cytokine profiling of cultured lymphocytes

A.2.1 Methods from CD4+ T cells enrichment and stimulation media

A.2.1.1 Preparation of media

Media A: RPMI 1640, 5 mM HEPES, 2 mM glutamine, 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco).

Media B: RPMI 1640, 5 mM HEPES, 2mM glutamine, 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanal, 10 mM sodium pyruvate, 1 mM non-essential amino acids (Gibco).

A.2.1.2 Preparation of feeder cells using mitomycin C

One day prior to isolation of CD4 T cells, spleen cells from naïve mouse were isolated and incubated with mitomycin C (CAT#M4287; Sigma, St. Louis, MO), 0.5 mg/2 ml/spleen in media A, at 37 ºC for 30 min. After 3 times wash with media A, these cells were kept in media A for culture at 37 ºC/5% CO_2 overnight. Cells were then counted [a].

A.2.1.3 Enrichment of CD4+ T cells

1. About ~0.125ml/spleen of Dynabeads mouse CD4 (L3T4; CAT#114.45; Dynal Biotech ASA, Oslo, Norway) was mixed with 0.25 ml of Media A in a 15 ml conical tube. The beads were then pooled down to the bottom of the tube by using magnetic particle concentrator (Dynal MPC-1). The supernatant was then discarded and the cells were resuspended in 0.125 ml of Media A.

2. Spleens were removed from immunized mice, washed 3 times with HBSS, and then homogenized into individual cells by pushing through a cell strainer (70 μm, BD Falcon).

3. Magnetic beads prepared in step 1 were added to the spleen cells (~2 ml in Media A) and incubated at 4 ºC for ~20 min, rotating.

4. Supernatants were discarded and the cells were washed 3 times with Media A.

5. The cells were then resuspended in 0.125 ml of Media A and added with 25 μl of DetachaBEAD mouse CD4 (CAT#124.06; Dynal Biotech ASA, Oslo, Norway). The mixture was then incubated for one hour at room temperature, rotating.

6. Supernatants were collected by using the magnetic particle concentrator. The cells were washed 3 times with Media A and all washes were pooled together with supernatants. Collected cells were counted [b], centrifuged and resuspended in Media B in proper cell concentration.

A.2.1.4 Concentration of cells and reagents [c].

1. CD4+ T cells: 4×10^5 /well were mixed with feeder cells at 1×10^6 /well.

2. For positive control, concanavalin A (Con A; 10 μg/ml) was added to mixed CD4+ T cells and feeder cells.

3. For GFP stimulation, anti-CD28 antibody (50 μg/ml, R&D Systems) was added into each well together with GFP (dialyzed in PBS; 25 μg/ml; Clontech). Anti-CD28 antibody without GFP was used as the media-alone control.

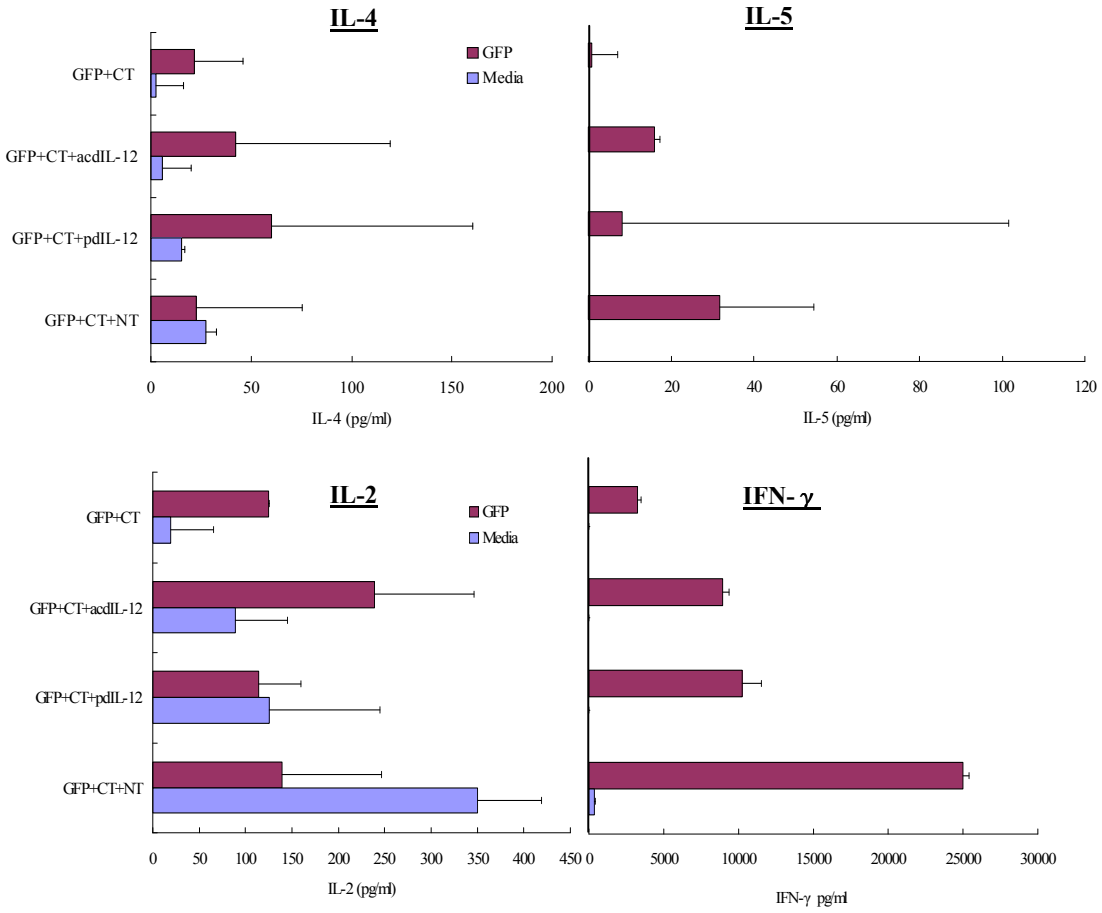
A.2.1.5 Cytokine profiling

After 3 days culture, the supernatants were collected for Th1/Th2 cytokine assays (CAT#171-F11081, Bio-Rad) by using Bio-Plex suspension array system (Bio-Rad, Hercules, CA) following manufacturer's protocols.

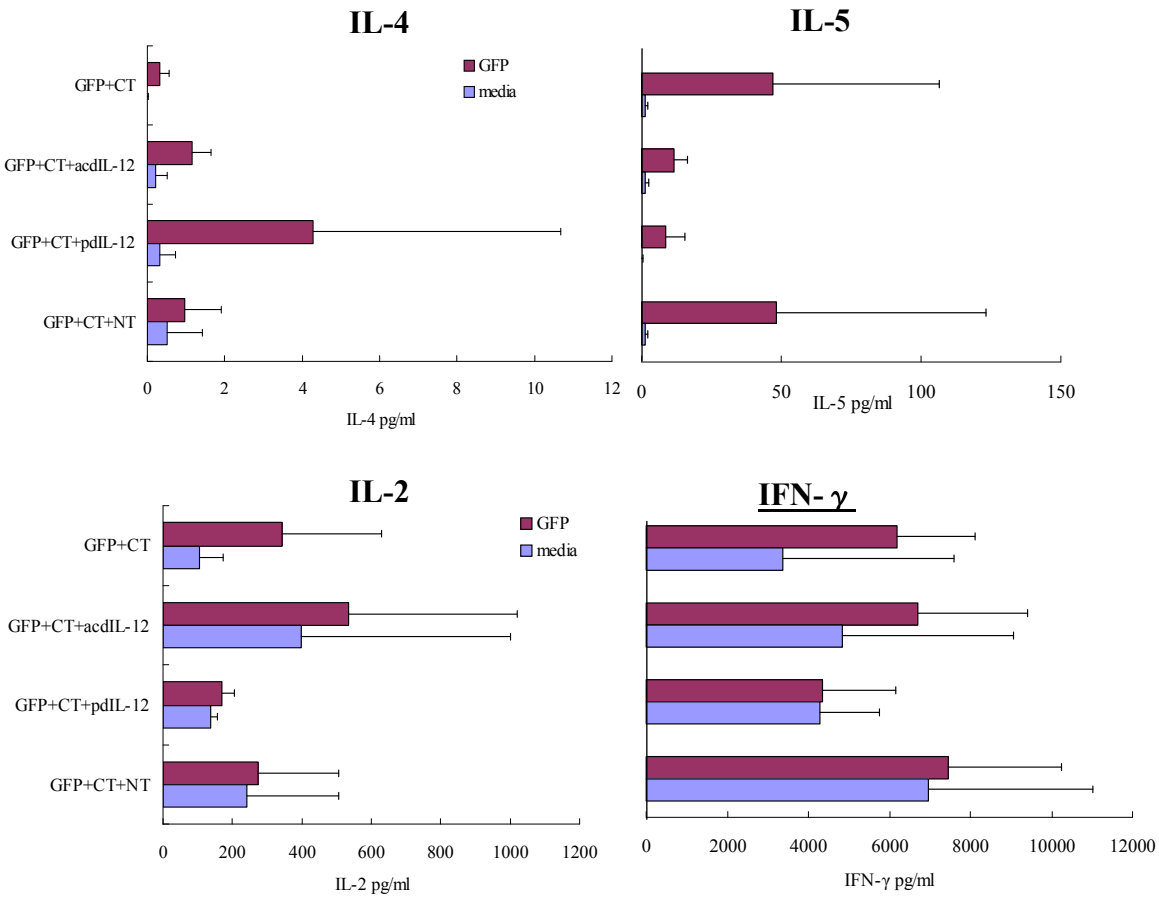
[a] About 1×10^8 cells can be recovered as feeder cells from each spleen.

[b] About 5×10^6 to 3×10^7 CD4+ T cells can be enriched from each spleen.

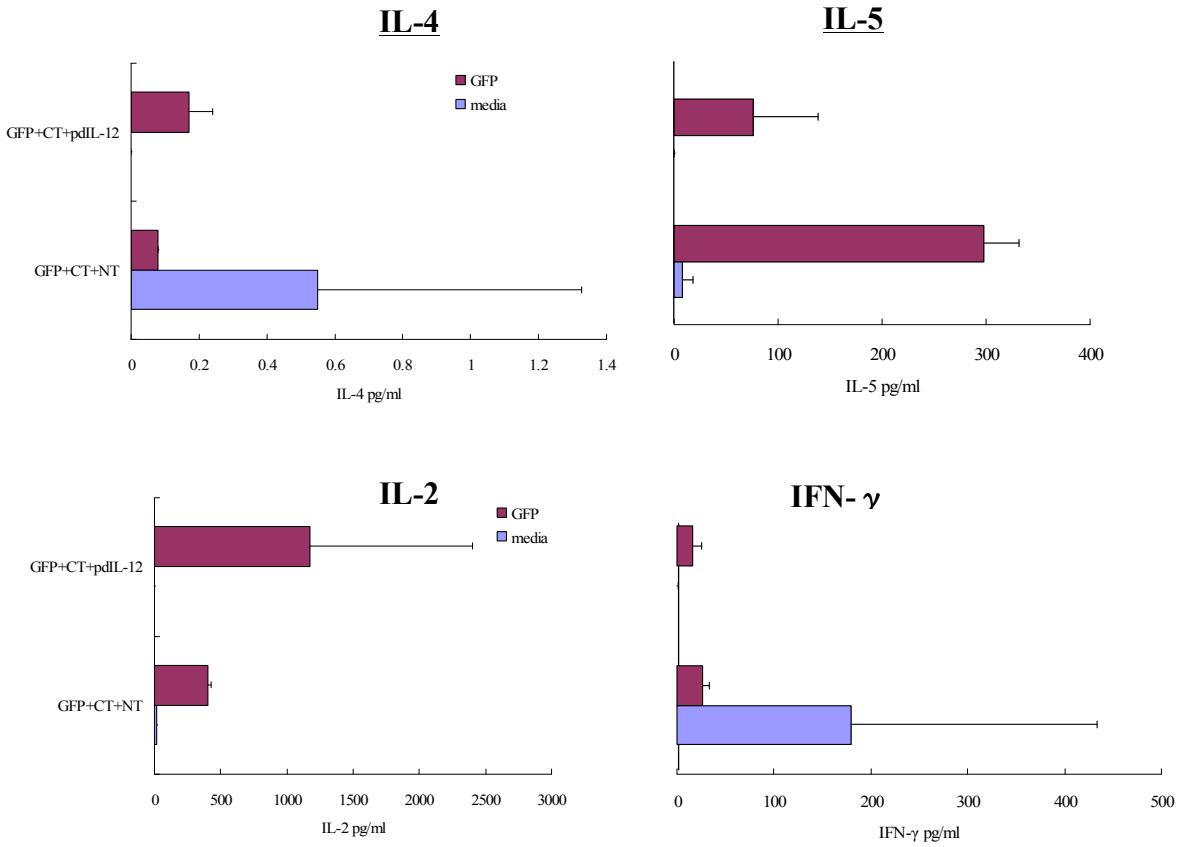
[c] The culture is 200 μl/well in Media B.



A.2.2 Cytokine secretion from cultured spleen cells. In trial 1, groups of ICR mice were immunized by GFP alone, or with the presence of CT, with or without animal-cell-derived IL-12 (acdIL-12), plant-derived IL-12 (pdIL-12), or non-transgenic control (NT) in week 1,3 and 5 (described in Chapter 3). One week after the final immunization, three mice from each group were sacrificed and spleen cells were isolated, pooled and cultured with (plum) or without (periwinkle) GFP (25 μg/ml) for 3 days. Cell culture Supernatants were collected for cytokine assays. Bars represent the mean level of cytokines +1 SE of duplicates.



A.2.3 Cytokine secretion from cultured lymph node cells. In trial 2, groups of ICR mice were immunized by GFP alone, or with the presence of CT, with or without animal-cell-derived IL-12 (acdIL-12), plant-derived IL-12 (pdIL-12), or non-transgenic control (NT) in week 1, 3 and 5 (described in Chapter 3). One week after the final immunization, 6 mice from each group were sacrificed and cervical lymph node (CLN) cells were isolated. CLN cells from 2 mice were pooled in each group, and cultured with (plum) or without (periwinkle) GFP (25 μg/ml) for 3 days. Cell culture Supernatants were collected for cytokine assays. Bars represent the mean level of cytokines +1 SE for 6 mice in each group.



A.2.4 Cytokines secretion from enriched CD4+ T cells. In Chapter 3, two groups of ICR mice were immunized by GFP, CT and plant-derived IL-12 (pdIL-12), or non-transgenic control (NT) in week 1, 3 and 5. One week after the final immunization, 2 mice from each group were sacrificed and spleen cells were isolated. CD4+ T cells were enriched as described in Appendix 2.1 and cultured with (plum) or without (periwinkle) GFP (25 μg/ml) for 3 days with the presence of co-stimulator anti-CD28 antibody (50 μg/ml). Supernatants of cell culture were collected for cytokine assays. Bars represent the mean level of cytokines +1 SE of 2 mice in each group.

Vitae

Jianyun (Jean) Liu was born on October 26, 1976 in Dongtai, Jiangsu Province, China. She graduated from Lanzhou University with a B.S. degree in Biochemistry in 1998. In the following four years, she worked as a QC technician in a pharmaceutical company and a project assistant in a biotechnology company in China. In 2002, she entered graduate school as a Ph. D student under the supervision of Drs. Carole Cramer and Craig Nessler in the Department of Plant Pathology, Physiology and Weed Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.