**Allergen-specific subcutaneous immunotherapy in allergic asthma: immunologic mechanisms and improvement**

Yousef A. Taher1*, Paul A.J. Henricks2 and Antoon J.M. van Oosterhout3

1Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Al-Fateh Medical University, Tripoli, Libya; 2Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands; 3Laboratory of Allergology and Pulmonary Diseases, University Medical Center Groningen, Groningen University, Groningen, The Netherlands

Allergic asthma is a disease characterized by persistent allergen-driven airway inflammation, remodeling, and airway hyperresponsiveness. CD4+ T-cells, especially T-helper type 2 cells, play a critical role in orchestrating the disease process through the release of the cytokines IL-4, IL-5, and IL-13. Allergen-specific immunotherapy (SIT) is currently the only treatment with a long-term effect via modifying the natural course of allergy by interfering with the underlying immunological mechanisms. However, although SIT is effective in allergic rhinitis and insect venom allergy, in allergic asthma it seldom results in complete alleviation of the symptoms. Improvement of SIT is needed to enhance its efficacy in asthmatic patients. Herein, the immunoregulatory mechanisms underlying the beneficial effects of SIT are discussed with the ultimate aim to improve its treatment efficacy.

Keywords: allergic asthma; immunotherapy; dendritic cell; regulatory T cells; Th2 lymphocytes; hyperresponsiveness; eosinophilia; IgE; IL-10

Allergic asthma is recognized as a chronic inflammatory disease of the airways in which many types of cells play a role, in particular mast cells, eosinophils, B- and T-lymphocytes, and epithelial cells. It is characterized by the production of allergen-specific immunoglobulin (Ig)E antibodies, reversible airflow obstruction, airway hyperresponsiveness (AHR) to a wide variety of specific or non-specific stimuli, chronic airway inflammation, and airway remodeling (1). During the past several decades, the prevalence of allergic asthma is increasing dramatically (>20% in every 10 years), especially in children (2). Approximately, it affects 10–15% of the population in most developed industrial countries (2). Both genetic and environmental factors are involved in allergic asthma. Genetic studies indicate that multiple genes are linked to the onset of this disease (3–5). However, the epidemic increase of asthma cannot be explained by genetic changes in the population. Changes in environment and lifestyle including isolation of houses which increases numbers of house dust mite, and childhood immunizations have contributed to the increase (6–9). In addition, epidemiological and clinical studies have suggested a link between the relative decreased exposure to bacteria or other pathogenic agents (such as helminthes) during the first years of life, and the increase in allergic asthma (10–12); this is referred to as the ‘hygiene hypothesis.’

**Pathogenesis of asthma**

The concepts of the pathogenesis of allergic asthma are illustrated in Fig. 1. The development of allergic asthma requires sensitization to an environmental allergen, which can occurs years before the onset of clinical symptoms (6, 13). For allergic sensitization allergens are taken up and processed by professional antigen-presenting cells (APCs), such as dendritic cells (DCs) and presented to allergen-specific T-cells that subsequently differentiate into T-helper (Th) 2-type cells. Activation of Th2 cells
leads to the production of inflammatory cytokines, such as interleukin 4 (IL-4) and IL-13, thereby stimulating allergen-specific B-cells to proliferate and switch to production of IgE (14, 15). Then IgE binds to high-affinity IgE receptor FcɛRI on mast cells. Upon re-exposure, the allergen will bind to allergen-specific IgE on mast cells thereby cross-linking the IgE-FcɛRI complexes which subsequently cause the release of preformed mediators like histamine and several other inflammatory mediators (16, 17). These mast cell-derived mediators cause the early asthmatic reaction which is characterized by airway smooth muscle constriction, extensive vascular leakage, mucus hypersecretion, enhanced airway responsiveness, and recruitment of inflammatory cells (17–19).

In approximately 50% of the asthmatic patients this early phase reaction is followed by the late phase reaction. This phase is characterized by significant involvement of infiltrated inflammatory cells, such as eosinophils, T lymphocytes and macrophages, and resident epithelial, endothelial and smooth muscle cells in promoting the chronic symptoms of airway inflammation. In addition, these cells may also be an important source of inflammatory mediators like chemokines, cytokines, and leukotrienes in asthma.

Asthma: a CD4⁺ T-helper type 2 (Th2)-mediated disease

CD4⁺ T-cells play a crucial role in controlling inflammation in allergic asthma. They are the predominant lymphocyte population that infiltrates the airways in asthmatics and are activated in these sites, expressing the surface activation markers class II histocompatibility antigen [HLA-DR], CD25 (IL-2R), and very late activation antigen-1 (VLA-1) (20, 21). In addition to CD4⁺ T-cells, CD8⁺ T-cells, and γ/δ T-cells have been identified in the airways of allergic asthmatics (21). About 60% of the CD4⁺ T-cells in the airway of persons with asthma are invariant natural killer T (iNKT) cells (22–25). However, there is still controversy whether iNKT-cells can induce allergic asthma in the absence of CD4⁺ T-cells (26–28).

Human and murine CD4⁺ T-cells are divided into two broad functional subsets according to their profiles of cytokine secretion (29, 30). The conditions under which CD4⁺ Th cells become activated during an immune response determine the development toward the Th1- or the Th2-phenotype (31). CD4⁺ Th1-type cells secrete predominantly IL-2 and interferon gamma (IFN-γ) and are particularly implicated in cell-mediated immune responses against invading intracellular pathogens, such as viruses (32, 33). CD4⁺ Th2-type cells produce a different panel of cytokines including IL-4, IL-13, and IL-5 (34). IL-4 promotes the development of Th2 cells and together with other cytokines, promotes the growth of mast cells, basophils, and eosinophils (35, 36). Furthermore, IL-4 and IL-13 are required for the induction of proliferation and isotype switching to IgE by B-cells that recognize the allergen (14, 37). IL-5 secretion by Th2 cells is critical for eosinophil differentiation and maturation (38, 39).

Fig. 1. Schematic representation of the pathophysiology of allergic asthma. In genetically predisposed individuals, primary exposure to an allergen leads to activation of Th2 lymphocytes and stimulation of IgE synthesis. Later on, exposure causes immediate release of the biologically active mediators (histamine, leukotrienes) via mast cell degranulation and further activation of Th2 cells with resulting eosinophil inflammation and AHR.

Citation: Libyan J Med 2010, 5: 5303 - DOI: 10.3402/ljm.v5i0.5303
Over the years it became clear that the allergen-induced airway inflammation is orchestrated by activated Th2 cells. Th2-type lymphocytes play a critical role in the initiation, progression, and persistence of allergic asthma. Initially, a disturbed balance between Th1- and Th2-mediated immune responses has been postulated to underlie aberrant Th2 reactions to ‘innocuous’ environmental antigens. Indeed, allergen-specific T-cell clones isolated from the blood of allergic individuals express a Th2 cytokine profile secreting IL-4, IL-5, and minimal IFN-γ and IL-2, whereas those clones from non-atopic individuals displayed a Th1 profile (40). Furthermore, allergic asthma is associated with the expression of IL-3, IL-4, and IL-5 in bronchoalveolar cells, strongly supporting Th2 activation (41). Moreover, it has been shown that when antigen-specific Th2 cells activated with inhaled antigen, Th2 cells-induced airway eosinophilia, mucus hypersecretion, and AHR after short-exposure to antigen, while Th1 cells resulted in a neutrophil-predominant inflammatory response without mucus production or AHR (42, 43), indicating that Th1 cells alone do not produce any of the characteristic features of asthma. In human studies, CD4+ T-cells producing IL-4, IL-5, and IL-13 have been identified in bronchoalveolar lavage fluid and airway biopsies from asthmatics patients. These cytokines are secreted in the Airways of patients with mild or asymptomatic disease (41). Furthermore, it has been shown that CD4+ Th2 lymphocytes are increased in the Airways of asthmatic patients after antigen challenge (21, 44, 45).

Allergen-specific immunotherapy (SIT)

Current pharmacologic therapies for allergic asthma, such as bronchodilators and inhaled corticosteroids, are effective in reducing and preventing symptom development, but do not reverse the progression of or cure this disease. SIT is unique in that it not only reduces symptoms, but also induces long-lasting disease remission. SIT has been shown to improve allergic dysfunction and to re-direct the immune system away from the allergic response.

SIT was first introduced at St. Mary’s Hospital London, at the end of the nineteenth century and many of the basic principles described remain valid today. In 1911, Noon carried out the first study of active immunization to prevent allergy against grass pollen using s.c. injection of a distilled water extract of the pollen of timothy grass, *Phleum pretense* (46). In 1918, it was generally accepted that hay fever, asthma, and anaphylaxis were the result from antibodies produced after exposure to sensitizing antigen (47).

By 1961, a new technique was developed to allow *in vitro* measurements of histamine release from cells in whole blood in the presence of specific allergen before and after treatment with ragweed extract (48). The findings demonstrated that after SIT, histamine release was completely abolished in a few patients and was reduced in others. Five years later, a double-blind study performed by Lichtenstein and Osler (49) demonstrated that the treatment with crude ragweed extract or the major allergen of ragweed ‘Amb a 1’ resulted in reduction of cellular sensitivity in some patients. Treated patients showed little correlation between cellular sensitivity and symptom scores. This finding was accompanied with a rise in blocking antibodies. After these results, the term immunotherapy has been used to describe the process because it greatly deals with complex immunologic changes.

**Specific immunotherapy (SIT) for allergic asthma**

SIT for the management of allergic asthma continues to be a matter for discussion (50–53). Abramson and colleagues (51, 54, 55) performed a meta-analysis of all trials published over the past 50 years of the twentieth century, examining the impact of SIT in patients with allergic asthma. Overall, SIT was efficacious of decreasing asthma medication use, reducing bronchial hyperresponsiveness, and improving asthma symptom scores. While there was no consistent effect on lung function, SIT significantly reduced the Airways response to inhalation of specific allergen, with some reduction in non-specific AHR as well.

**Mechanisms involved in specific immunotherapy (SIT)**

The potential mechanisms that have been proposed to explain the beneficial effects of SIT are illustrated in Fig. 2. To explain the immunological mechanisms underlying the clinical improvement, intensive research has concentrated upon the specific antibody response in serum with respect to class and subclass distribution (56). Studies showed that the allergen-specific IgE levels rise temporarily during initial phase of SIT, but fall back to pre-treatment levels during maintenance therapy (57). Subsequently, it was demonstrated that SIT also induces allergen-specific IgG (mostly IgG1 and IgG4) and in few reported cases IgA levels (58–60). These findings led to the hypotheses that these IgG antibodies contribute to the immunosuppressive effects of SIT by blocking IgE-facilitated antigen presentation (61, 62) and that such antibodies act as blocking antibodies by engaging low-affinity Fc receptors for Ig (e.g. FcyRII) expressed by B lymphocytes, basophils, and mast cells (60). In fact, a substantial number of studies demonstrated increased specific IgG4 levels together with clinical improvement (63, 64). However, the role of IgG (and IgA) in SIT has been questioned and it is not clear whether the significant increase in IgG levels has a causal role in alleviating symptoms or simply represents a bystander effect.
occurring as a consequence of high allergen exposure. In Fcy knockout mouse model of allergic asthma we found that bronchoalveolar lavage (BAL) eosinophilia, Th2 cytokines, and serum ovalbumin (OVA)-specific IgE levels were strongly suppressed by SIT, demonstrating that IgG Fcy receptors have no major role in the beneficial effects of SIT (unpublished data). Moreover, results demonstrated that the suppression of allergen-induced airway inflammation by SIT is not B-cell dependent, ruling out a critical role for B-cells, IgGs, and IgA in SIT in the mouse model of allergic asthma.

Thus, in the context of allergic diseases, including asthma, most research indicates that during SIT the increase of IgG is an epiphenomenon, however, the role of allergen-specific antibodies, as putative ‘blocking factors(s),’ in SIT providing protection against allergic disease appears unlikely.

**T-cell tolerance**

The generation of an effective immune response involves antigen-specific T-cell expansion and differentiation of effector function. T-cell activation requires at least two distinct signals, including signaling via the antigen-specific T-cell receptor and a co-stimulatory pathway (31). Antigen stimulation of T-cells can lead either to a positive immune response, characterized by proliferation, differentiation, clonal expansion, and effector function, or in the absence of an appropriate co-stimulatory signal to a state of long-lasting unresponsiveness, termed anergy (65). The most important co-stimulatory molecules involved are CD28/CD152 (CTLA-4)–CD80/CD86 (B7-1/-2) and CD40-CD154 (CD40 ligand) (66–68).

Anergy induction can down-regulate both cellular and humoral immune responses. The induction of T-cell non-responsiveness or anergy as the mechanism of SIT is supported by the observed diminution of allergen-specific T-cell proliferation and cytokine production (69), although in some instances IL-2 was able to restore these functions (70). However, the usually increased IgG production during SIT cannot be explained by the induction of T-cell anergy (71, 72). Various methods for anergy induction have been described. Stimulation of T-cells with high doses of antigen in the absence of appropriate co-stimulation or in the presence of IL-10 has been found to induce a profound form of anergy (73, 74). Under these conditions anergic T-cells showed limited peripheral expansion and a significant amount of death, with a residual subset of cells remaining that were unresponsive to antigen restimulation both in vivo and in vitro. It has been suggested that T-cells rendered anergic in vivo and in vitro become regulatory T-cells that can regulate other T-cell responses (75, 76).

**Immune deviation toward T-helper type 1 (Th1) responses**

In the late 1980s, a favorable explanation was based on the Th1/Th2 dichotomy in specific immune reactions.
Mechanistic studies demonstrated that SIT alters the balance of cytokines released from Th-lymphocytes with a shift from Th2-cells in association with allergic inflammation toward Th1-cells that release IFN-γ, which inhibits Th2-cells (77–79). Early studies showed that allergen-specific T-cell clones shift from IL-4 to IFN-γ production after SIT (77, 80). However, the effects of SIT on Th1 cytokine secretion are less consistent (81). In humans, several clinical trials demonstrated a reduction of the allergen-specific Th2 response, whereas the Th1 response remained undetectable (78, 82) and in a separate study, SIT diminished both allergen-specific Th2 and Th1 responses (83). In experimental allergic asthma models some studies have confirmed that the relation between responses (83). In experimental allergic asthma models some studies have confirmed that the relation between Th1 response by SIT lost favor with the alternative recent years, the concept of immune deviation toward disease, but others have not found such effects (84). In recent years, the concept of immune deviation toward Th1 response by SIT lost favor with the alternative observation on regulatory T-cells.

Adaptive regulatory T (Treg) cells are taking the center stage as crucial immunoregulatory cells that may offer an explanation for many of the observations that occur during SIT that cannot be adequately explained by the Th2 to Th1 shift (85–87).

Regulatory T-cells
Several subsets of regulatory T-cells with distinct phenotypes and mechanism of actions have been identified. These include: (1) naturally occurring CD4+CD25+Foxp3+ T-cells (nTregs) that inhibit immune responses through cell-to-cell contact, (2) CD4+Foxp3+ iTregs induced in the periphery, in contrast to nTregs not generated in the thymus; the capacity of these cells to suppress the proliferation of naïve T-cells requires cell–cell contact, and (3) cells induced in the periphery following antigen exposure (aTreg cells). aTreg cells are subdivided into type 1 regulatory T (Tr1) cells which secrete high levels of IL-10 and low to moderate levels of transforming growth factor (TGF)-β and type 3 regulatory T (Th3) cells which secrete TGF-β.

Natural CD4+CD25+Foxp3+ T regulatory cells
The natural CD4+CD25+Foxp3+ regulatory T (nTreg) cells are generated in the thymus and represent 5–10% of the CD4+ T lymphocytes both in mice and humans. These cells are thought to perform a specialized role in controlling both the innate and the adaptive immune response (88, 89). Depletion of nTreg cells leads to the spontaneous development of various autoimmune diseases (90–96). Although the mechanism of immune regulation mediated by nTreg cells is not well understood, these cells can both directly suppress responding T-cells and down-modulate APC function in vitro (97, 98). nTreg cells may also convert CD4+ Th cell into regulatory cells expressing IL-10 and/or TGF-β in culture systems (99, 100). It has been shown that nTreg cells induce infectious tolerance in vivo by catalyzing the formation of IL-10 producing regulatory T-cells (101).

Adaptive T regulatory cells
In recent years, it has been demonstrated that SIT is associated with the induction of aTreg cells. The aTreg cells are generated in the periphery from naïve T-cells after encountering antigens presented by tolerogenic DCs. In studies of SIT for bee venom anaphylaxis, Akdis et al. (85) were the first to provide evidence for a role of IL-10 and Tr1 cells in the beneficial effects of SIT. They observed that the therapy-induced increase in secretion of IL-10 in T-cell cultures along with decreased allergen-driven proliferation and decreased production of Th2 and Th1 cytokines. Moreover, in clinical trials, SIT has been shown to increase the production of IL-10 by APCs, including B cells, monocytes, and macrophages (61, 87, 102). Findings were extended by SIT studies with airborne allergens that showed increases in IL-10 and TGF-β producing antigen-specific Treg cells in the blood and airway tissue (61, 85–87). Induction of IL-10 producing Tr1 cells has been shown to be associated with successful SIT (85, 86). Animal studies confirmed the suppressive role of IL-10 during allergic inflammatory diseases (84, 103–106). In human studies, the regulatory cytokine IL-10 has been shown to increase the production of IgG4 while preventing IL-4-mediated class switching to IgE (107). Furthermore, IL-10 down-regulate IgE-dependent activation of basophils and mast cells, and decrease survival and activation of eosinophils (108). In addition, IL-10 (and TGF-β) might act on APCs. IL-10 down-regulates Major Histocompatibility Complex (MHC) class II expression on monocytes and reduces their antigen presentation capacity (109). Moreover, IL-10 down-regulates the CD80 expression on DCs and macrophages (110, 111). IL-10 may therefore block the APC dependent CD28–CD80 interaction and subsequent co-stimulatory signaling in T-cells (112).

The other immunoregulatory cytokine associated with regulatory T-cells is TGF-β. Studies have provided evidence for increases in the amount of TGF-β-driven allergen-specific IgA following SIT, indicating other antibody classes than IgG might contribute to clinical efficacy (87). Moreover, TGF-β has been shown to induce the expression of IL-10 by T-cells (113). Literature data suggest that early increase in IL-10 (85) and sustained TGF-β production may be required for successful SIT to induce stable tolerance to non-pathogenic environmental antigens (114).
Role of dendritic cells (DCs) in generation of adaptive regulatory T (aTreg) cells

Although there is suggestive evidence that SIT achieves beneficial effectiveness by the induction of aTreg cells, the mechanism by which SIT induces aTreg cells is not completely understood. Over the past decade, it has become clear that DCs play a critical role in the generation of all aTreg cell subsets (115) and regulation of immune responses to a variety of antigens (116). The ability of DCs to induce immunity or tolerance depends on their maturation state. Tolerogenic DCs are immature cells with increased expression of MHC II and CD86 but low levels of expression of CD40 and absence of the pro-inflammatory cytokines IL-6 and TNF-α (117). It has been shown that aTreg cells induced by immature DCs are characterized by high levels of IL-10 cytokine secretion (118, 119). The nuclear factor-kB (NF-kB) protein Re1B activity is critically required for DCs maturation (120). Re1B-deficient bone marrow-derived DCs (BMDCs) or BMDCs in which Re1B activity is inhibited have the potential to induce antigen-specific immune tolerance in vitro (121). Inhibition of NF-kB signaling by various drugs (122–124) induces DCs to acquire tolerogenic properties that favor the induction of Tr1-like cells in vitro and tolerance in mouse models of transplantation and autoimmune diseases (125–127).

Besides this, recent findings demonstrate that DCs induce development of aTreg cells by several mechanisms including high expression of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) (128, 129). IDO expressing DCs inhibit T-cell proliferation in vitro and promote tolerance in vivo (130, 131), including maternal tolerance during pregnancy (132), control of allograft rejection (133), and protection against autoimmunity (134). A number of studies have also demonstrated that IDO is implicated in tolerance induction by regulatory T-cells expressing cytotoxic T-lymphocyte antigen-4 (CTLA-4) (135, 136). Moreover, IDO may directly mediate inhibition of T-cells proliferation by tryptophan depletion or downstream tryptophan metabolites (137, 138). Recently, we showed that inhibition of IDO throughout SIT abrogated the efficacy of SIT on the reduction of BAL eosinophil numbers and AHR to methacholine, but no changes in serum OVA-specific IgE levels were observed (139). In addition, we demonstrated that inhibition of IDO throughout the aerosol challenge period did not interfere with tolerance induction by SIT. These findings reveal that IDO plays a role only during induction of tolerance by SIT and that activity of IDO is irrelevant for the effects of SIT thereafter. Moreover, considering the conceptual possibilities that either tryptophan depletion or downstream tryptophan metabolites mediated-IDO immunoregulation we showed that formation of tryptophan metabolites, rather than tryptophan depletion, is the mechanism by which SIT induces tolerance to the induction of airway manifestations of asthma.

Improvement of specific immunotherapy (SIT)

The goal of SIT is the transformation of an allergic individual into one who can tolerate allergen exposure by converting deleterious allergic immune response into protective responses. Despite the impressive efficacy of allergen (injection) SIT for treatment of allergic rhinitis and insect venom allergy, its efficacy in allergic asthma remains controversial (51). Furthermore, it has been reported that s.c. administration of allergens can induce severe systemic reactions due to cross-linking of allergen-specific IgE on mast cells (140, 141). Thus, for development of a more effective and safe form of SIT, more insight into the underlying immunological mechanisms of allergen IT is considered necessary to improve efficacy, in particular in asthmatic patients. There is growing evidence that the positive effects of SIT are associated with aTreg cells (Tr1 and Th3) and the immunosuppressive cytokines that they produce. The mechanisms by which these cells are induced by SIT are still not fully understood. Well-described pathways to induce aTreg cells are antigen presentation to T-cell by immature DCs or the presence of IL-10 or TGF-β in the local microenvironment. In this regard, combination of SIT with administration of compounds that inhibit DCs maturation, such as the biologically active form of vitamin D₃ (1,25-dihydroxyvitamin D₃) or glucocorticoids, might be novel immunotherapeutic strategies to improve SIT for better treatment of allergic diseases, including allergic asthma. Interestingly, in a mouse model of allergen immunotherapy, we recently demonstrated that co-administration of 1,25-dihydroxyvitamin D₃ effectively suppressed AHR to methacholine and potentiated the reduction of serum allergen-specific IgE levels and BAL eosinophilia in the suboptimal SIT regime matched by a reduction in Th2 cytokines, IL-5, and IL-13 (142). We further observed that in vitro antigen-induced productions of Th2 cytokines are decreased in lung-draining lymph node cells after this combination SIT. Moreover, data demonstrates that the immunoregulatory cytokines IL-10 and TGF-β are involved in tolerance to allergen-induced airway manifestation of asthma since the suppressive effects of combined SIT are completely abrogated by blocking of the IL-10R and neutralizing of TGF-β by using mAbs at the time of antigen inhalation challenge.

Conclusion

Peripheral (naturally occurring and adaptive) T-cell tolerance is the key immunological mechanism in
healthy immune response to self- and non-infectious non-self-antigen. Changes in the balance between allergen-specific Treg and Th2, and/or Th1 cells are very crucial in the development and also treatment of allergic diseases. Understanding of the immunological mechanisms that lead to tolerance induction by SIT, Fig. 3, in particular the role of regulatory T-cells in allergen-specific peripheral tolerance may lead to more rational and safer approaches that could result in prevention and cure of allergic diseases, including asthma.

Acknowledgements

This study was partly financially supported by the Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands.

Conflict of interest and funding

Y.A.T was supported by a grant 965.02 from the Libyan Ministry of Higher Education.

References

Immunotherapy in allergic asthma


52. Bousquet J. Pro: immunotherapy is clinically indicated in the management of allergic asthma. Am J Respir Crit Care Med. 2001; 164: 2139-40. (Discussion 41-2)

53. Adkinson NF Jr. Con: immunotherapy is not clinically indicated in the management of allergic asthma. Am J Respir Crit Care Med. 2001; 164: 2140-1. (Discussion 1-2)


Immunotherapy in allergic asthma


131. Yousef A. Taher
Department of Pharmacology and Clinical Pharmacy
Faculty of Pharmacy
Al-Fateh Medical University
P.O. Box 83001, Central Post
Tripoli, Libya
Tel: +218 92 3022980
Fax: +218 21 4625577
Email: ymadane@yahoo.co.uk

Citation: Libyan J Med 2010, 5: S303 - DOI: 10.3402/ljm.v5i0.5303