CHAPTER 4

EFFECT OF INDUKANTHA GHRITHA ON TH1 and TH2 TYPE IMMUNE RESPONSE
4.1. INTRODUCTION

Cytokines, a large group of soluble extracellular proteins or glycoproteins, are key intercellular regulators and mobilizers. Classified into family groups (e.g., interleukins, interferons, and chemokines) based on the structural homologies of their receptors, these proteins were initially believed to act primarily as antiviral (Young AS and Cummins JM, 1990) or antineoplastic (Terlikowski SJ, 2001) agents. They are now seen to be crucial to innate and adaptive inflammatory responses, cell growth and differentiation, cell death, angiogenesis and developmental as well as repair processes (Oppenheim JJ, 2001). Their secretion, by virtually every nucleated cell type, is usually an inducible response to injurious stimuli. In addition, cytokines provide a link between organ systems, providing molecular cues for maintaining physiological stability (O’Sullivan RL, 1998).

The immune system plays a crucial role in maintenance of the body homeostasis and ability of committed Th1 and Th2 cells to function in altered cytokine environments is a central issue in autoimmune and immune-mediated diseases. CD4 (helper) cells are mainly responsible for orchestrating and directing an immune response. IL-2, IFN-γ, IL-12 are major cytokines produced by Th1 cells, contributing to cell mediated immune response while IL-4 and IL-5 are secreted by Th2 cells, mediating the humoral responses (Morgan H et al, 2006). It has been shown that the Th1 cytokine, IFN-γ promotes immunoglobulin switching from IgM to the IgG2a isotype. At the same time, IL-4 triggers switch from IgM to IgG1 and IgE. Indeed, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses. The relative production of these isotypes can thus be used as a marker for the induction of Th1-like and Th2-like immune responses, respectively. Th1 and Th2 cytokines also function to cross-regulate Ig isotypes (Finkelman FD et al, 1990), (Paul WE, 1870). The mononuclear phagocyte system (macrophages, monocytes), dendritic cells, lymphocytes (T and B cells) and natural killer cells provide an important role in inflammation, repair, cellular and humoral immunity and metabolic and neoplastic disease processes (Andrew DL et al, 2005).

GM-CSF and MIP-1α, promote the recruitment, activation, and/or differentiation of dendritic cells and macrophages. Activated dendritic cells and
macrophages secrete IL-12 and/or other factors that stimulate NK cells and T cells to secrete IFN-γ (Aderem and Underhill, 1999). Several studies demonstrate MIP-1α to play a significant role in phagocytic activation (Al-Mokdad et al, 1998) capable of inhibiting HIV-1 infection in susceptible cells and tumor development (Crittenden M et al, 2003).

Treatment with recombinant cytokines has yielded a number of adverse effects, such as transient lymphopenias induced by IFN, IL-2, and TNF. Monocytopenia has been reported with the use of interferon gamma (IFN-γ) and TNF, while IL-2, IFN-γ, and TNF induce neutrophilia (Engel A et al, 1994). Patient experience of flu-like symptoms with the use of interferons makes adherence to a therapeutic protocol a challenge. Both IL-2 and IFN-γ, used for the treatment of hepatitis C and some cancers, are known to evoke depression, fatigue, sleepiness, irritability, and loss of appetite (Wichers M and Maes M, 2002). These toxic side effects have limited the clinical value of such therapies (Belardelli F and Ferrantini M, 2002).

In light of the adverse events experienced with cytokine-targeted therapy, it could prove useful to consider the use of phytotherapy in the modulation of cytokine expression. Immune-related illnesses have long been treated with herbal medicines. The primary literature suggests many of the effects of botanicals may be via cytokine modulation (Spelman K, 2002). The term immunomodulator has been used in the phytotherapy literature to describe botanical medicines believed to influence immunity (Spelman K, 2000). With regard to phytotherapy, immunomodulators may be defined as botanical medicines that alter the activities of the immune system via the dynamic regulation of informational molecules—cytokines, hormones, neurotransmitters, and other peptides.

Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases. One strategy in the modulation of cytokine expression may be through the use of herbal medicines. A class of herbal medicines, known as immunomodulators, alters the activity of immune function through the dynamic regulation of informational molecules such as cytokines. The in vitro and in vivo research demonstrates that the reviewed botanical medicines modulate the secretion
of multiple cytokines. The reported therapeutic success of these plants by traditional cultures and modern clinicians may be partially due to their effects on cytokines. Phytotherapy offers a potential therapeutic modality for the treatment of many differing conditions involving cytokines.

4.2. MATERIALS AND METHODS

Mouse immunoglobulin ELISA kits

- IgG1 (cat no:E90-105)
- IgG2a (E90-107)  
  Bethyl Laboratories Inc. U.S.A

Mouse cytokine ELISA kits

- mouse IL-2 (KMC0021)
- mouse MIP1α (KMC2201)  
  Biosource International Inc, USA
- GM-CSF (KMC2011)
- IFNγ (BMS606)
- IL-12 (BMS616)  
  Bender Med systems, CA
- IL-4 (BMS613)

All other chemical used were of analytical reagent grade.

4.2.1. Experimental Animals

As described previously in chapter 3

4.2.2. IgG subclass analysis

The level of IgG1, IgG2a, in serum of both control and IG administered animals were measured by enzyme-linked immunosorbent assay (ELISA). All reagents and incubation periods were at room temperature to enhance assay precision and accuracy. Certified microtiter plates were coated with rat anti-mouse IgG1 or IgG2a in phosphate buffered saline (PBS), pH 7.3 sealed and incubated overnight at 4°C. The plates were washed 3 times between each incubation with PBS + 0.5% Tween 20. To block nonspecific binding, 200µl of PBS + 1% BSA was added to plates. After 30 min plates were washed as described above and mouse serum and appropriate purified mouse immunoglobulin standards were applied. Following one hour incubation, washed as described above, biotinylated rat antimouse IgG1 or
IgG2a in blocking buffer was added to all wells. After 1 hour incubation and 3 washes, streptavidin-horseradish peroxidase conjugate was added and the plates incubated for 20min. Following 3 washes, 3, 5'- 5,5' tetramethyl benzidine substrate solution(TMB) was added and incubated for at least 20 min. Optical density was read on a ELISA plate reader at 450nm.

4.2.3. Cytokine evaluation

To investigate the effect of IG on various cytokine levels of IL-2, IFNγ, IL-12, IL-4, MIP1α and GM-CSF were performed in the serum of control and IG treated animals by ELISA method.

For cytokine production, 1X 10^6 cells/well of whole spleen cells were cultured in RPMI-1640 medium containing 5% FBS with PHA (10μg/ml) at 37°C for 48h in 5% CO₂. After the stimulation, the levels of IL-12, IFNγ, IL-2 and IL-4 in culture supernatant were determined by commercially available ELISA kit. The tests were performed according to supplier’s instruction manual.

Diluted samples or IL-2, IFNγ, IL-12, IL-4, MIP1α and GM-CSF standards were added in duplicate. The plates were incubated for 2 hour at room temperature. The supernatant was discarded and the wells were washed 5 times with wash buffer. Subsequently Avidin-HRP conjugated secondary antibody was added and incubated for 1hour at room temperature. After washing tetramethyl benzidine substrate solution was added. The color was allowed to develop for 30 minutes in the dark before the reaction was quenched with a stop solution (0.2M H₂SO₄).The plates were read at 450nm and sample concentrations were determined with the help of a standard curve.

4.2.4. Statistical Analysis

The results were expressed as the mean ± S.D. The differences between the control and the treatment in these experiments were evaluated for statistical significance by using Student “t” test.

4.3. RESULTS

4.3.1. Measurement of Serum Immunoglobulin levels

After 14 days of IG administration, sera from individual mice were tested for IgG1, IgG2a isotypes by ELISA. We thus measured antibody isotypes in the serum,
using IgG2a as a measure of type 1 responses, and IgG1 as an indicator of type 2 responses. Although we observed no statistically significant difference in the amount of circulating immunoglobulin levels of IgM and IgA during IG administration, a significant reduction in IgG1 and IgE was seen in IG administered animals. In addition IgG2a level was found to be significantly increased in IG treated animals in a dose dependant manner. The IgG1/IgG2a antibody ratios thus supported the cytokine data and demonstrated that IG administered animals were able to induce Th1 type immune response. IG elicited higher levels of IgG2a than IgG1, indicating a predominant Th1 response, which is known to be crucial for a complete bacterial clearance and is able to provide protective immunity against intracellular pathogens.

4.3.2. Cytokine evaluation

To investigate if IG modulates Th1 and Th2 cytokine profile in mice we performed cytokine analysis of IL-2, IL-12, IFNγ and IL-4 in culture supernatant of splenocytes after 48 hours of incubation. Culture supernatant from IG treated mice produced significantly increased level of IL-2, IFNγ, IL-12 indicating that IG can enhance Th1-type immune response in vivo. In contrast IL-4 production was slightly decreased after oral administration of IG. These results indicate that the Th cell responses in Balb/c mice were shifted to the Th1-dominant state by IG administration (p ≤ 0.05). These results suggest that IG has an ability to suppress the Th2 response in a dose dependant manner.

![Graph](image_url)

Figure 4.2. The capacity of lymphocytes to be stimulated in vitro and to produce cytokines was confirmed in the presence of phytohemaglutinin after 14 days of IG
treatment. Enhanced level of Th1 related cytokines like IL-2, IFN-γ, IL-12 and reduction in Th2 cytokine like IL-4 following treatment with IG for 14 days. (IG = Indukantha Ghritha).

The stimulating effect of oral administration of IG on the circulating levels of IL-2, IL-4, IL-12, IFN gamma, GM-CSF and MIP-1α in serum samples are shown in table 4.1. The results provide evidence for the involvement of MIP1α and GM-CSF in the rapid recruitment of immune cells. The increase in the circulating levels of these cytokines was significantly increased in IG treated mice. IL-2 and IFN gamma showed highly significant increase in IG treated animals supporting the increase in lymphocyte function seen in the proliferative assays. Significant increase was also seen in the concentration of IL-12 even though not to the extent as seen with IL-2 or IFN gamma. In contrast IL-4 production was slightly decreased after oral administration of IG.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>IL-2 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>GM-CSF (pg/ml)</th>
<th>MIP-1α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>88.3±5.2</td>
<td>289±10.0</td>
<td>29.1±1.40</td>
<td>95.35±7.4</td>
<td>48.3±3.2</td>
<td>11.8±0.9</td>
</tr>
<tr>
<td>3rd day</td>
<td>280±12.5**</td>
<td>322±25.6*</td>
<td>33.2±2.52</td>
<td>92.45±8.6</td>
<td>120.4±9.2**</td>
<td>14.7±1.20*</td>
</tr>
<tr>
<td>7th day</td>
<td>480±23.4**</td>
<td>426±28.3*</td>
<td>38.0±2.75*</td>
<td>81.91±4.1*</td>
<td>147.8±11.4**</td>
<td>18.9±1.5**</td>
</tr>
<tr>
<td>14th day</td>
<td>497±19.6**</td>
<td>519±24.6**</td>
<td>49.6±3.16**</td>
<td>67.07±5.5*</td>
<td>118.8±8.5**</td>
<td>20.0±1.2**</td>
</tr>
<tr>
<td>21st day</td>
<td>480±17.0**</td>
<td>502±31.8**</td>
<td>52.4±3.82**</td>
<td>68.36±4.3*</td>
<td>122.8±7.3**</td>
<td>20.0±1.1**</td>
</tr>
<tr>
<td>28th day</td>
<td>485±24.3**</td>
<td>511±27.3**</td>
<td>54.8±2.97**</td>
<td>70.27±6.1*</td>
<td>120.3±8.6**</td>
<td>20.1±1.4**</td>
</tr>
</tbody>
</table>

* P-value < 0.01 ** p-value < 0.001

Table 4.1. In vivo Cytokine levels in serum following administration of 250mg/kg of IG.
Figure 4.1. Serum IgG subclass IgG1 (A), IgG2a (B) titers were determined 14 days of IG administration at various doses ranging from 30mg/kg to 1000mg/kg body weight. Results are expressed as the mean ± SD of 8 individual mice. Immunoglobulin levels were determined by ELISA method. Significance were determined for IG administered animals vs controls * p ≤ 0.05 ** p ≤ 0.01. (IG = Indukantha Ghritha).
4.4. DISCUSSION

Cytokines operate both as a cascade and as a network, regulating the production of other cytokines and cytokine receptors, while stimulating the production of acute-phase proteins (Gabay C and Kushner I., 1999). Endogenous levels of cytokines are in nanomolar to picomolar range, suggesting that dilute mixtures of biologically active compounds may provide therapeutic benefit. Illustrating the therapeutic potential for dilute mixtures of biologically active compounds, a group of researchers found that subclinical doses of oral IFN-γ can provide powerful, broad-spectrum benefits. In another study, when cytokine levels were compared to symptoms in individuals with cardiovascular disease, Testa et al (1996) demonstrated that circulating levels of cytokines increased with severity of symptoms. Considering the variety of adverse events listed for recombinant cytokine therapies, perhaps subtle perturbations of the cytokine network should be considered. The dilute nature of botanical immunomodulators may offer a reasonable strategy for subtle induction of a variety of cytokines. Most likely, cells are seldom exposed to only a single cytokine. Rather, combinations of cytokines and other messenger molecules generate biologically relevant informational cues. This is demonstrated by the synergic antitumor effects observed from combining IL-12 gene therapy with other cytokines, chemokines, or co-stimulatory molecules (Belardelli F and Ferrantini M, 2002). The effects of cytokines on their target cells and tissues may be inhibited or enhanced by other cytokines, hormones, and cytokine-receptor antagonists and circulating receptors. Just as pharmacological activity by specific plant constituents is suggested to be affected by combinations of constituents, (Spelman K et al, 2006), (Gilbert B and Alves LF, 2003), (Wills RB et al, 2000) combinations of cytokines have been found to have additive, inhibitory, or synergic effects (Gabay C and Kushner I, 1999). Further research may find that the herbal immunomodulators affecting multiple cytokines can each generate a unique signature of immune perturbation dependent on the concerted effect on arrays of cytokines.

Changes in immune function determines health, even life and death because every single cell in an organism is in one way or another in communication with circulating and tissue resident effector cells of the immune system (Fuleihan RL,
2005). Cytokines are required for activating and inactivating as well as deleting cells of the immune system during immune responses (Nakajima H and Takatsu K., 2006). No data are available to date on immunomodulatory property of IG, cytokine profile in vivo. Our results showed that IG increased CD4 cell number with concurrent increase in IL-2, IL-12, IFN-γ with concentrations ranging from 30mg/kg to 1000mg/kg. Although the mechanisms of its action and the active components in IG are unclear, the components present in IG are capable of acting on multiple targets to induce immune response. We hypothesize that IG exert significant immunomodulatory effects by differentially regulating the production of Th1 and Th2 derived cytokines. Hence this drug is useful to relieve symptoms that include low blood components production, general dryness, weakness, and low immune system functions, which may be results of radiation/chemo injuries, or side effects in treatment for cancers or AIDS. IG may seem to specifically target T helper cells. The end result of this immune modulation is an increase in Th1 related cytokines, increased lymphocyte proliferation and enhancement of NK cell number. Hence the current study focuses on the possibility that IG can contribute to the treatment and prevention of various diseases by the modification of cytokine level and T cell subpopulation and their functions.

Th1 and Th2 responses are known to favor IgG2a and IgG1 isotype switching, respectively (Estes DM and Teale JM, 1991). During IG administration elevations of IFN-γ and decrease in IL-4 in BALB/c mice suggest T-cell activation of type 1 subsets. The reduction in IgG1 and elevations in IgG2a levels suggest that these cytokine perturbations were functionally significant, since IL-4 and IFN-γ act as isotype switch factors for the B-cell production of IgG1 and IgG2a (Alizadeh M et al, 2006). This could reflect the enhanced IFN-γ observed in IG treated group because IFN-γ can have inhibitory effects on isotype switching to IgG1. The elicited higher levels of IgG2a than IgG1, indicating a predominant Th1 response, which is known to be crucial for a complete bacterial clearance and is able to provide protective immunity against intracellular pathogens (Takahashi N et al, 2006). Determining the Th1/Th2 cytokine profile induced by Indukantha Ghritha and IgG isotype response has been used to identify IG as a promising candidate of immune modifier.
Currently available data strongly suggest that IG may be a promising candidate as an immune modifier that maintains the homeostasis of immune function. IG seems to specifically target T helper cells. This immunomodulatory drug may be of value in the prophylactic treatment of several diseases involving severe defect in Th1 type immune response.