

## CHAPTER II

### LITERATURE REVIEW

#### 2.1. The Immune System

The Immune system is a form of body defense against the antigens such as infectious agents and malignancies. This system recognizes, processes, and then eradicates antigens. Based on the specification and memory, the immune system defense is classified into two arms, the innate and adaptive immunity. Both the systems share the coordination and develop a well-adjusted mechanism to eradicate the pathogens.<sup>1</sup>

Innate immunity is known as non-specific immunity. It is defined inasmuch of its ability to eradicate antigens at first line without previously exposed to this specific antigen. This type of immune system is consisted of epithelial barriers, molecules (complement system, C-reactive proteins) and cells of innate immunity such as NK cell, neutrophils, basophils, eosinophils, and monocytes-macrophages. Epithelial barriers are mucosal lining or skin that cover the body and protect with their intact lining. They are the very first barriers that need to be passed by the antigens before invading the host. Complement system consists of soluble proteins that interact one to another resulting in microbial cell wall lysis.<sup>3</sup> C-reactive proteins (CRPs) are the same soluble proteins, which are secreted by the liver in response to macrophages cytokines interact with the cell

walls of several pathogens such as C-polysaccharides of *Pneumococcus (Streptococcus pneumoniae)*.<sup>15</sup>

Phagocytes of innate immunity are the first cellular responses that encounter the pathogens. The cells are equipped with the pattern recognition receptors (PRRs), phagocytic capability, intracellular killing and some of them are able to present the antigens to the other immune cells, or also known as Antigen-Presenting cells (APCs). The PRRs will recognize part of microbes known as Pathogen Associated Molecular Patterns (PAMPs). The ability of phagocytes to phagocytize the pathogens is essential for the host, an infection can be prevented from dissemination and the APCs function will furthermore activate the adaptive immunity with the more augmented mechanisms.<sup>2,4</sup>

Adaptive immunity is a specified immunity where antigen-specification is needed and the secondary response becomes stronger. Memory is the other important factor that causes the response enhancement in adaptive immunity. There are two majors of defense based on the work site, cellular mediated and humoral mediated adaptive immunity.<sup>17</sup>

The cellular mediated adaptive immunity is mainly controlled by the coordination of APCs, adaptive T helper cells and effector cells. Antigen presenting cells are dendritic cells (conventional dendritic cells and plasmacytoid dendritic cells), monocytes, macrophages, and residential macrophages (Kupffer cells, Microglia, etc.). T helper cells are CD4<sup>+</sup> T-lymphocytes that undergo differentiation into currently known there major T helpers, such as T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 lymphocytes. T<sub>H</sub>1 lymphocytes are to activate macrophages to ingest and

digest the microbes. The  $T_H2$  lymphocytes function is to stimulate immunoglobulin E (IgE) antibodies and eosinophils mediated reactions particularly in helminthic infections. The  $T_H17$  cells secrete cytokines that recruits the neutrophils to the site of infection. The effector cells are macrophages and  $CD8^+$  T-lymphocytes/Cytotoxic T Lymphocytes (CTLs). Macrophages act on phagocytosis, and with the  $T_H1$  cells activation will enhance their phagocytosis activity. CTLs work particularly on the viral infected cells and malignant cells via the cytolytic activity that lyses the infected cells.<sup>18</sup>

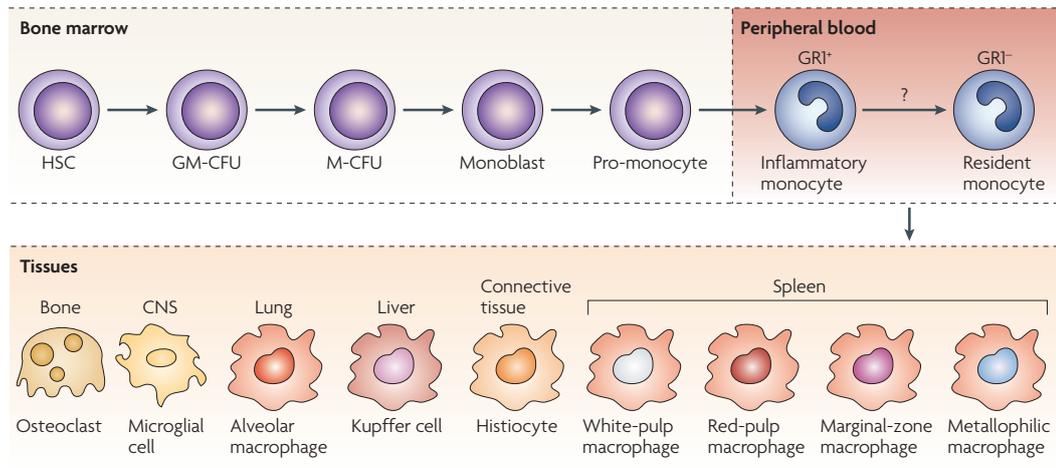
The molecules in body fluids, such as immunoglobulin (antibodies) and also the complement system, mediate the humoral adaptive immunity. The antibodies are secreted by the plasma cells, which are differentiated from B-cells.

## **2.2. The Role of Macrophages in Immune System**

Mononuclear phagocytic system (MPS) consists of bone-marrow-derived cells that have different morphologies and are mainly responsible for phagocytosis, cytokine secretion and antigen presentation. The cells include in this MPS are dendritic cells, monocytes and macrophages.<sup>2</sup>

Macrophages are derived from monoblastic lineage cells of bone marrow hemopoietic stem cells (HPSCs). Stem cells maturation requires stem cells factor, Flt3 ligand, and Interleukin-6 (IL-6). The mature stem cells are induced to turn into common myeloid progenitor via myelopoiesis.

The Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and IL-6 induce the common myeloid progenitor cells in turn to be monoblasts. This process is also involving the role of FLt3 ligand as well.



**Figure 1.** Mononuclear phagocytes develop in the bone marrow, circulate in the blood as monocytes, and are resident in all tissues of the body as macrophages. They may differentiate into specialized forms in particular tissues.  
Cited from Mosser<sup>18</sup>

The monoblasts are induced by Monocyte-Colony Stimulating Factors (M-CSFs) to transform into monocytes. Monocytes circulate in the blood and lymph circulation, where they can move to the tissues, transform or activated as macrophages and also undergo differentiation into residential macrophages.<sup>18</sup>

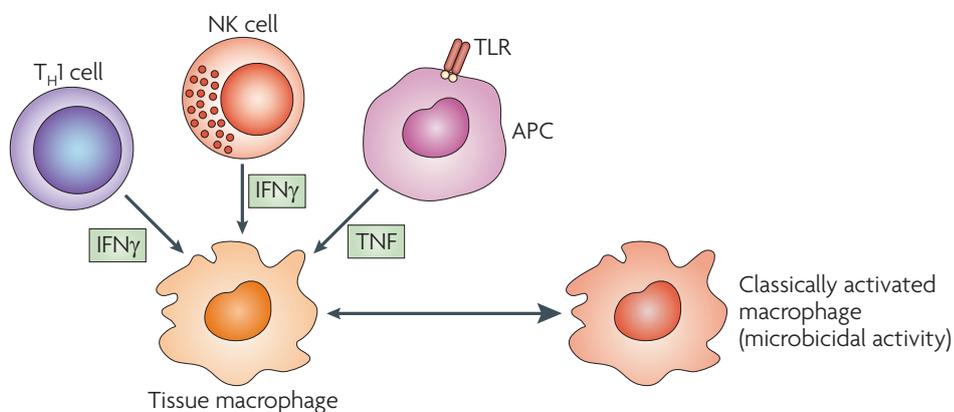
Some residential macrophages are known such as microglial cells (central nervous system macrophages), Kupffer cells (liver tissue macrophages), alveolar macrophages (lung tissue macrophages), peritoneal macrophages, and osteoclasts (bone tissue multinucleated cells that regulate bone reabsorption).

Macrophages have three main functions. They are phagocytosis, antigen presentation and cytokine production. The phagocytosis function is supported by the Pattern Recognition Receptors on the surface of macrophages such as Toll-like receptors, C-type lectin-like receptors, and scavenger receptors. Toll-like receptors identify various microbial molecules including bacterial lipopolysaccharide (LPS), peptidoglycans, and viral nucleic acids. Mannose

receptors functionally correlated to microbial surface carbohydrate. Scavenger receptors recognize microbial diacylglycerides.<sup>3</sup>

Macrophages possess receptors for carbohydrates that are not normally exposed on the cells of vertebrates, such as mannose, and therefore can discriminate between “foreign” and “self” molecules. In addition, both macrophages and neutrophils have receptors for antibodies and complement, thus the microbes antibodies or complement opsonization enhances phagocytosis.<sup>19,20</sup> The engulfed microorganisms are subjected to a wide range of toxic intracellular molecules, including superoxide anion, hydroxyl radicals, hypochlorous acid, nitric oxide, antimicrobial cationic proteins and peptides, and lysozyme.<sup>6</sup>

Antigen presenting cells are dendritic cells, macrophages and the B cells. Macrophages act both at the peripheral and central lymphoid tissues as the antigen presenting cells. Macrophages ingest the microbes, process them by the Major Histocompatibility Complexes (MHCs) and introduce them to the specific  $T_H1$  cells. Some cytokines secreted by specific  $T_H1$  cells such as  $IFN-\gamma$  will activate the effector macrophages.<sup>17</sup>



**Figure 2.**  $CD4^+$  T lymphocyte and NK cell activate Macrophage via secretion of Interferon gamma  
Cited from Mosser<sup>18</sup>

Activated macrophages secrete cytokines such as Tumor Necrosis Factor (TNF), Interleukin-1 (IL-1), Interleukin-12 (IL-12) and various chemokines. During phagocytosis, macrophages increase the class II MHC expressions to induce the recognition of T<sub>H</sub>1 cells.

In cellular mediated immunity, T<sub>H</sub>1 cells have role to activate macrophages as the effector cells. T<sub>H</sub>1 cells differentiation is stimulated mainly by the IL-12 and IFN- $\gamma$  in response to microbes and NK cells. Many intracellular microbes such as *Mycobacteria* species, *Salmonella typhi*, *Listeria monocytogenes*, and parasites such as *Leishmania* stimulate T<sub>H</sub>1 cells activation.<sup>2</sup> Antigen-Presenting Cell phagocytizes the microbe/antigen and introduces it to the subset of T<sub>H</sub>0 cell via the class II MHC. Antigen recognition process is followed by differentiation into T<sub>H</sub>1 cells.

Production of certain cytokines, including IL-12, Interleukin-18 (IL-18) and IFN- $\gamma$  promotes T<sub>H</sub>0 cells differentiation into T<sub>H</sub>1 cells. IL-12, secreted by macrophages, and dendritic cells primarily works in this mechanism along with IL-18 cytokine.

### 2.3. Immunostimulator

Immunostimulators (which is also known as immunostimulants, immunomodulators, etc.) are substances that augment, stimulate, activate, potentiate or modulate the immune response at either cellular or humoral level. The mechanism of action can be non-specific, resulting in the enhancement of the immune system responsiveness to antigens or related to the adjuvanticity.<sup>14</sup>

Flavonoids are one of the secondary active metabolites of various plants. The flavonoids possess medicinal effects and one of the effects is as immunostimulator. The mechanism of actions of Flavonoid in interfering with immune cells is based on variety of studies in vitro and in vivo. They are such as affecting secretory processes, mitogenesis, cells interactions, and probably the adhesion molecules expression. The gene expression is also represented with the secretion of cytokines that are major activators of immune system orchestra.<sup>21</sup> NF- $\kappa$ B (Nuclear Factor Kappa B), a transcriptional factor for IFN- $\gamma$  gene expression is activated via TNF-Receptor (Tumor Necrosis Factor-Receptor) results in IFN- $\gamma$  release.<sup>9</sup>

The molecular mechanisms of flavonoid as the immunostimulator are known via various mechanisms. One of the best explanations is via the modulation of the NF- $\kappa$ B system. NF- $\kappa$ B system modulate various cytokines secretion and postulated as the most possible track for flavonoid to modulate the immune system.<sup>9, 22</sup> Cellular measurements (Macrophages activity, NK cell activity, and lymphocytes count) and concentration of cytokines (IL-12, IL-18, and IFN- $\gamma$ ) are also intended to support proposed mechanisms.<sup>8-13</sup>

## 2.4. *Phaleria macrocarpa* (Scheff.) Boerl

### 2.4.1. Biology of *Phaleria macrocarpa* (Scheff.) Boerl

*Phaleria macrocarpa* (Scheff.) Boerl (Thymelaceae) is a tropical plant that exists in Southeast Asian archipelago that predominantly originates from Papua Island. The plant grows in the tropical area through ages and is one of the popular medicinal plants in Indonesia. Local people recognize *Phaleria* as ‘Pau’, ‘Makutodewo’, ‘Mahkota Dewa’ means ‘Crown of God’. Various active secondary metabolites such as flavonoids, alkaloid, terpenoid and saponin are available in the carp of fruit.<sup>23</sup>



#### Taxonomy of *Phaleria macrocarpa*

Kingdom	: <i>Plantae</i>
Division	: <i>Magnoliophyta</i>
Class	: <i>Magnoliopsida</i>
Order	: <i>Malvales</i>
Family	: <i>Thymelaeaceae</i>
Genus	: <i>Phaleria</i>
Species	: <i>Phaleria macrocarpa</i>

**Figure 3.** *Phaleria macrocarpa* (Scheff.) Boerl

In centuries, people of Indonesian Archipelago have discovered the function of *Phaleria* as empiric-based or traditional-herb therapy to treat cancer, hypertension, diabetes mellitus, and also acts as diuretic. Conferring to these facts, studies of new alternate medications derived from plant (phytopharmacology researches) are required. Flavonoid is one of metabolite components, classified under phenolic groups in *Phaleria*. It was proposed that the flavonoid of *Phaleria* gives effects to immunological effects.<sup>8,9</sup>

#### 2.4.2. Mechanism of Actions of *Phaleria macrocarpa* Extract

The mechanism of actions of flavonoids can be classified into antimicrobial mechanism and immunological mechanism. The antimicrobial mechanism is consisted of several routes, such as nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism that lead to pathogens direct elimination.<sup>23</sup> The immunological mechanism is described from the functions of flavonoids that affect gene expression and effects of cytokines and cytokine receptors. How all these effects are mediated is not yet clear, but one important mechanism may be capacity of flavonoids to stimulate or inhibit protein phosphorylation and thereby regulate cell function.<sup>21, 22</sup>

A study-conducted administration of the *Phaleria macrocarpa* leaf extract significantly increased killing activity of splenic NK 1.1 cells against targets. The effects are based on the secretion of IFN- $\gamma$  from NK 1.1 cells, and expression of surface molecules. During infections, NK cells will produce IFN- $\gamma$  rapidly before the development of adaptive immune response, approximately 4 hours after stimulation with *Phaleria* extract. And after 24 hours almost majority of NK cells are IFN- $\gamma$  positive. IFN- $\gamma$  is a potent activator of monocytes that allow macrophages to kill tumor cells and ingest microbes.<sup>8</sup>

Another suggested mechanism is by flavonoid binding to the TNF family receptor triggering the I $\kappa$ B kinases complex to phosphorylate I $\kappa$ B. The NF- $\kappa$ B proteins translocates to the nucleus and bind the DNA binding sites inducing transcription of gene for the IFN- $\gamma$ , T<sub>H</sub>1 immune response is activated and cellular mediated immunity orchestrated.<sup>9</sup>

## 2.5. *Phyllanthus niruri* Linnaeus

### 2.5.1. Biology of *Phyllanthus niruri* Linnaeus

*Phyllanthus niruri* (Indonesian: *meniran*) is one of the popular herbs with historical and long-usage in herbal medicine systems such as Ayurveda Medicine (India), Traditional Chinese Medicine and Jamu (Indonesia). The whole plant is used as remedies of lots conditions such as dysentery, influenza, vaginitis, tumors, diabetes, diuretics, jaundice, kidney stones and dyspepsia. Meniran has extensive diversity of phytochemicals and pharmacological properties. The active phytochemicals (secondary metabolites) such as flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins have been identified from various parts of *Phyllanthus niruri* Linn.<sup>25</sup>

#### Taxonomy of *Phyllanthus niruri* Linnaeus



Kingdom	: <i>Plantae</i>
Division	: <i>Spermatophyta</i>
Sub Division	: <i>Angiospermae</i>
Class	: <i>Dicotyledonae</i>
Order	: <i>Euphorbiales</i>
Family	: <i>Euphorbiaceae</i>
Genus	: <i>Phyllanthus</i>
Species	: <i>Phyllanthus niruri</i> Linn

Figure 4. *Phyllanthus niruri* Linn.

Isolated the three active metabolites from *Phyllanthus* which actively inhibit the hepatitis B viral replication, increase the immune system capabilities and protect hepatocytes.<sup>26</sup> The flavonoids of herbal plants such as *Phyllanthus*

*niruri* increase the activity and function from both cellular and humoral component of immune system.<sup>11</sup>

### **2.5.2. Mechanism of Actions of *Phyllanthus niruri* Extract**

The preclinical test with mice covered both safety and immunomodulatory characteristic of *Phyllanthus*. It is obtained *Phyllanthus* extract modulates immune system via proliferation and activation of the T-cells and B-cells, secretion of specified interleukins such as IFN- $\gamma$ , tumor necrosis factor alpha (TNF- $\alpha$ ), interleukins (ILs), complement system activation, and phagocytes activation such as macrophages and monocytes.<sup>19</sup>

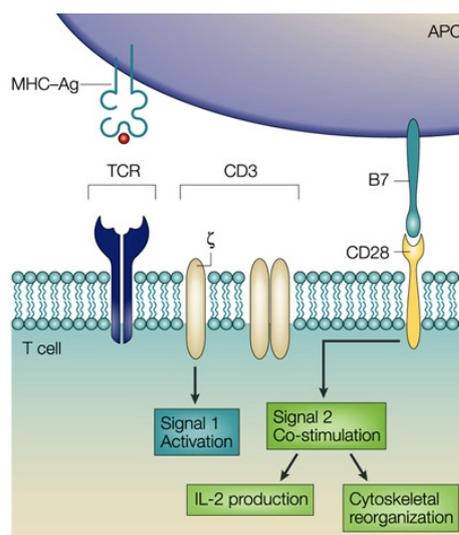
In another pathway, CTLs activity is also increased, also NK cells activity. Clinical trial was also conducted to measure immunomodulatory effect of *Phyllanthus niruri* to several patients with various conditions. The immunomodulatory effect suggests the usage as adjuvant therapy for several infectious diseases.<sup>27, 28</sup>

The increase of IL-12 production by Bone Marrow-derived Dendritic cells (BM DCs) under the *Phyllanthus niruri* treatment.<sup>10</sup> IL-12 is secreted by dendritic cells and macrophages and stimulates IFN- $\gamma$  production by NK cells and T cells enhance NK cells and CTLs-mediated cytotoxicity. This condition promotes differentiation of T<sub>H</sub>1 cells. IFN- $\gamma$  and IL-12 stimulate T<sub>H</sub>1 cells differentiation by activating the transcription factors T-bet, STAT1, and STAT4. Both cytokines stimulation increases IFN- $\gamma$  production. Increase of IFN- $\gamma$  production amplifies the T<sub>H</sub>1 cells to produce more IFN- $\gamma$ . It results in inhibition of T<sub>H</sub>2 cells, T<sub>H</sub>17

cells development, and carries effector function such as macrophage activation and production of some antibody isotypes.

*Phyllanthus niruri* also increases antigen-presenting functions of BM DCs. It is significantly shown from the higher proliferation for T cells which are antigenically presented by *Phyllanthus niruri* treated dendritic cells, compared to the non-treated group.<sup>10</sup>

IL-2 production was increased twofold higher by *Phyllanthus niruri* treatment in a co-culture of BM-DCs/CD8<sup>+</sup> T cells. LPS treatment to samples also strongly increased IL-2 production by T cells in culture supernatant. This result suggests that activation and maturation of BM-DCs by *Phyllanthus niruri* treatment resulted in enhancement of their APC functions to T cells.<sup>10</sup> The presence of higher expression of costimulators and increases secretion of cytokines IL-2 followed by proliferation and differentiation of T cells.



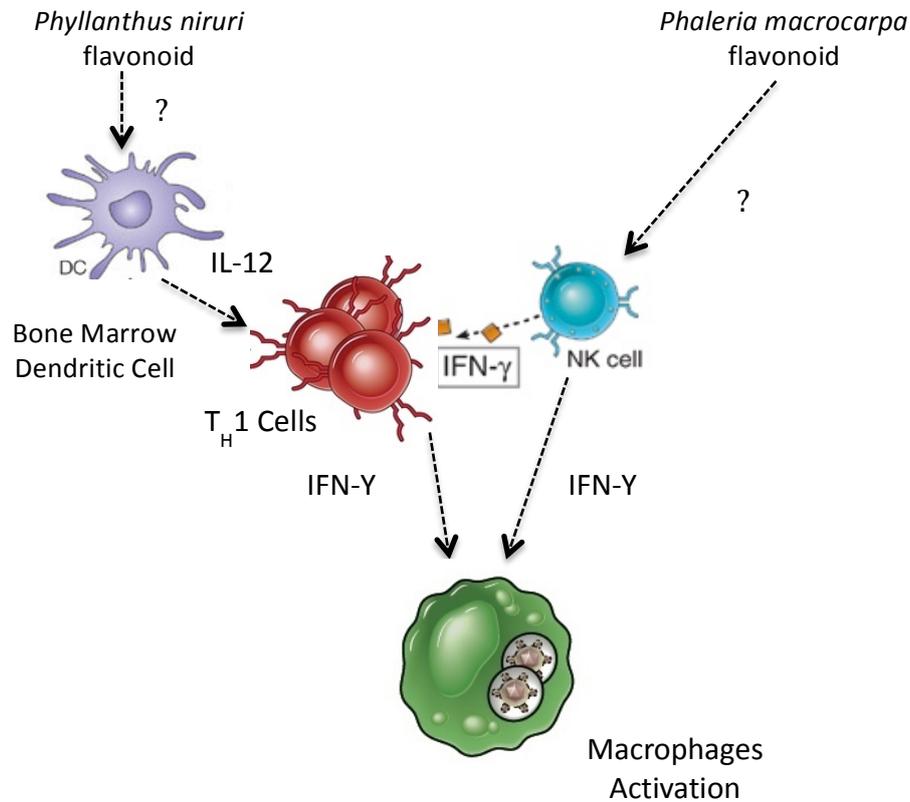
**Figure 5.** Antigen-Presenting Cell increases the expression of costimulator molecule and secretion of cytokines IL-2  
Cited from Sadelain<sup>29</sup>

A study showed the increase of immunologic parameter after the *Phyllanthus* treatment such as complement, IgM, IgG level, neutrophil chemotaxis, macrophage chemotaxis, T cells-B cells proliferation, NK cells cytotoxicity, CD8<sup>+</sup> T lymphocytes cytotoxicity, TNF- $\alpha$  and IFN- $\gamma$  level. In human there is an increase on Proliferation of B cells and T cells, neutrophil phagocytosis, NK cells cytotoxicity, and secretion of TNF- $\alpha$  and IFN- $\gamma$ .<sup>11,12</sup>

The other study also reported the administration of *Phyllanthus* extract increases immunologic status through increasing of lymphocytes infiltration and perforins expression in colon cancer.<sup>13</sup>

## **2.6. Effects of Combination of *Phaleria* and *Phyllanthus* Extract on Immune System**

This research is the first research conducted about the combination extracts hence there is not found any publication related to this issue. Both herbs contain the flavonoid metabolites, which have been proposed as immunostimulators. The mechanism of actions of *Phyllanthus niruri* in immune system is mainly by secretion of Interleukin-12.<sup>9</sup> This secretion will result in augmentation of T<sub>H</sub>1 cells proliferation, and activation followed by the IFN- $\gamma$  secretion. Macrophages activation occurs with the stimulation given from the IFN- $\gamma$ .



**Figure 6.** Summarized Mechanism of Actions of *Phaleria macrocarpa* and *Phyllanthus niruri* extracts on immune system  
 Illustraed based on researches of Ghufron, Budijitno, Nworu, et Maat<sup>8,9,10,11</sup>

The mechanism of actions of *Phaleria macrocarpa* in immune system is via stimulation of NK cells to secrete the IFN- $\gamma$ .<sup>8</sup> This leading to activation of macrophages as cellular mediated immunity effector cell. It was hypothesized the combination of both herbs will result in boost of immune system, or known as the synergistic mechanism.