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Review Article

From Immunosenescence to Immunocompetence by Use of Lipid Delivered Nano Gold - The Dynamics of an Oral Vaccine against SARS-CoV 2

Sharadendu Bali[1]* and Ram Parajiya[2]

[1]*Professor of General Surgery, MMIMSR, Ambala, India*

[2]*Resident in General Surgery, MMIMSR, Ambala, India*

***Corresponding Author:** Sharadendu Bali, Professor of General Surgery, MMIMSR, Ambala, India.*

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Abstract

Immunosenescence has been postulated by many workers to be the prime reason behind the increased mortality observed in the ageing population during the ongoing SARS-CoV 2 pandemic. Defects and dysfunctions of the primary immune cells are being considered as the most important causes of the hyper-inflammatory responses seen in the patients admitted to the ICU, and in those that succumb to this highly contagious disease. These defects may be in the efficient and early recognition of PAMPs by the PRRs of the Dendritic cells or in the delayed and hyper-inflammatory cytokine release by the monocytes and macrophages in the lung. The innate immune system response is now being advanced as the critical factor in disease outcome.

While Immunosenescence is considered irreversible, yet it is a dynamic process and as such it is amenable to corrections and upgradations. One agent that can revitalize the ageing immune system is Gold nanoparticles (GNP) which have recently been studied because of their ability to act as carriers of vaccines and chemotherapeutic agents. Toxicity studies conducted to assess the impact of these particles on immune cells have yielded some astonishing results. Use of GNP in rats has caused increase in lymphocyte cell populations and induced activation of dendritic cells and macrophages. Since immunosenescence causes a depletion of naive T and B lymphocytes, use of GNP can have a salutary role. Improved recognition of PAMPs by Dendritic cells and Macrophages can help in mounting a more efficient response to the invading virus.

Elemental gold formulations have been used in India for millennia, for diseases ranging from fevers and memory loss to senility. Some formulations employ honey and ghee (clarified butter) in combination with GNP. These formulations are prescribed for infants, hence their safety is not suspect. If employed with suitable modifications, such formulations can play the vital role of oral vaccines against not just Covid 19, but also the vast range of other influenza viruses.

Keywords: *Immunosenescence; Immunocompetence; Lipid; Oral Vaccine; SARS-CoV 2*

Abbreviations

IS: Immunosenescence; ICU: Intensive Care Unit; GNP: Gold Nano Particles; NK Cells: Natural Killer Cells; IL: Interleukin; SARS: Severe Acute Respiratory Syndrome; CoV: Corona Virus; IFN: Interferon; MALT: Mucosa Associated Lymphatic Tissue; PAMP: Pathogen Associated Molecular Patterns; PRR: Pathogen Recognition Receptors; TLR: Toll Like Receptors; HSC: Haematopoietic stem cells; CMP: Common Myeloid Progenitor; ALI: Acute Lung Injury; MHC: Major Histocompatibility Complex; CD: Cluster Differentiation; APC: Antigen Present-

ing Cells; BALF: Broncho: Alveolar Lavage Fluid; DC: Dendritic Cells; SGNP: Solid Gold Nano Particle; HGG: Honey, Ghee, Gold Suspension; SP: Swarn Prashan; GIT: Gastro Intestinal Tract

Introduction

The ongoing SARS-CoV 2 pandemic has highlighted the susceptibility of the older age group to the attack by the novel coronavirus. It is increasingly being recognized that immunosenescence is the underlying cause of the much greater mortality seen in the aged [1,2]. It is also clear that a shift towards immunological competence would greatly assist in decreasing the mortality from Covid-19. Ancient Indian Medicine has remedies for rejuvenation of the aged, as well as formulations to vaccinate infants orally against microbial attack. Many such preparations contain gold in nano-particle form. In recent years, Gold nanoparticles (GNP) have been found to have a powerful positive impact on Innate immunity, adaptive immunity and immunomodulation, besides being safe and biocompatible [3,4].

Enhancing the activity of the innate immune system has been proposed as a means to protect against SARS-CoV-2 [5]. Over the past few years, GNP have been found to have a profound effect on the activation and functions of primary immune cells [6,7]. The efficacy of formulations containing GNP as a means for re-invigorating the innate and adaptive immunity have immense potential for priming the immune system to combat Covid-19. If delivered orally in association with lipids, the potency of the formulation can be much enhanced by direct delivery to the intestinal lymphatics, and further to the cardio-pulmonary circulation, bypassing the portal circulation and thence the first-pass metabolic degradation in the liver [8]. Since the Novel Coronavirus primarily kills by affecting the lungs, stimulation and immuno-modulation of the immune cells residing in the mucosa associated lymphatic tissue (MALT) of the lungs may further amplify the protective role of GNP in combating this deadly disease.

The formulation described in ancient Indian Medicine Classics to immunize infants [9] consists of three components - GNP, honey and ghee (clarified butter). Raw honey contains a multitude of amino acids, glycosylated peptides, oligosaccharides, antigens and nucleotides. As we shall see, conjugation of these with GNP occurs spontaneously and results in a tremendous immune response. Ghee acts as a carrier and also participates in formation of lipid microspheres containing the GNP- peptide conjugates by virtue of its constituent phospholipids. The formulation is discussed scientifically as a means to combat immunosenescence and its potentiality to function as vaccine against Covid 19. Hereinafter, we shall refer to this formulation as HGG (honey-ghee-gold).

Properties of gold nanoparticles

Gold NPs are chemically inert and relatively stable. The excellent biocompatibility and low toxicity of GNP make them an important tool in bionanotechnology [10]. In aqueous solution, spherical GNPs show a range of colours, from brown, orange, red to purple, as the size increases from 1nm to 100nm. Gold NPs are customizable in their size and shape which has functional significances in immunotherapies. The size and shape of gold NPs may influence the ability of gold NPs to reach the targeted cells (*e.g.* cancer cells) and antigen-presenting cells (dendritic cells and macrophages), as well as their interaction capacity with these cells (internalization process) [11].

In addition, the functionality of gold NPs can be fine-tuned after surface functionalization with numerous molecules including antibodies, antigenic peptides, nucleic acids, polymers and radioisotopes [11]. The large surface energy and natural affinity of GNP allows them to easily form conjugates when they contact proteins, peptides, polysaccharides or lipids, and the latter are then known as capping agents [12]. This conjugation is due to covalent or non-covalent mechanisms like covalent bonding, physical adsorption, or weak electrostatic interactions. The term Biodecorated GNP is used for Gold NP conjugated with large number of other molecules or substances (Figure 1). More about this will be discussed in section *Rationale of Gold Sup*.

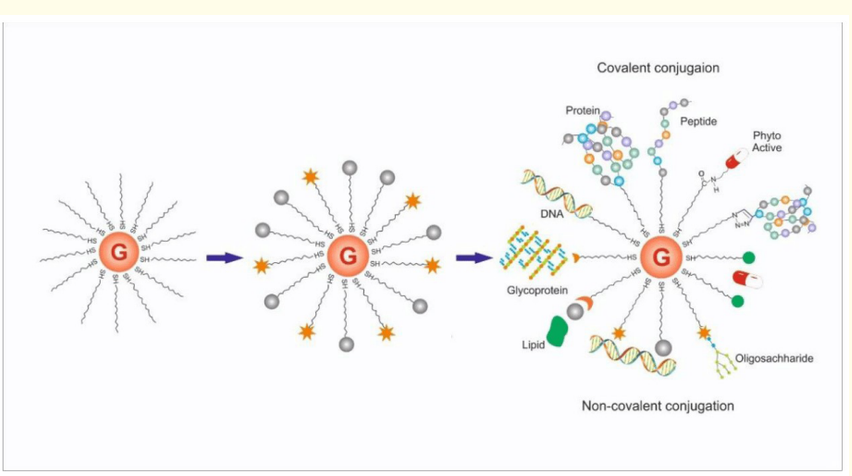

Figure 1: *Tethering of organic molecules and biomolecules to the surface of GNP through place ligand exchange.*

Use of GNP in immunotherapies and vaccines

GNPs preferentially accumulate in immune cells, as these cells are specialised to interact with foreign material. GNP are taken up by APCs by phagocytosis, pinocytosis or endocytosis, depending upon the size. GNPs have been utilized to deliver therapeutic compounds for immunomodulation, especially in auto-immune disorders and in cancer [13,14]. Numerous studies have suggested that GNPs conjugated with several functional molecules induce a robust immunity, as required by Vaccines [15]. Sonia Carabineiro in her paper has elucidated several dozen trials wherein GNP in association with viral antigens, engineered virus-like particles, plasmid DNA etc elicited strong immunological responses [12]. For example, gold NPs conjugated with M2e, the ectodomain of M2 protein present on influenza A virus, induced high levels of antibody response, providing complete protection against influenza virus challenge [16].

Immunosenescence

Immunosenescence (IS) refers to the gradual deterioration in the immune function brought on by advancing age. It affects both the innate and adaptive immune systems, though the latter is affected more. There is thus an increased susceptibility to infections and a decreased ability to develop immunity after immunization, resulting in higher morbidity and mortality in the elderly [17]. In brief, it is characterized by the following:

1. Diminished ability of haematopoietic stem cells (HSC) for self-renewal and a decline in function, associated with bias towards common myeloid progenitor (CMP) cells [18].

2. Cytotoxicity of Natural Killer (NK) cells is diminished, though the numbers are increased [19].

3. Impairment of the phagocytic capacity and reduced superoxide anion production in neutrophils [17]. Along with this, apoptosis of neutrophils is hindered (20).

4. Defective signalling of Pattern Recognition Receptors (PRR) in Dendritic Cells (DC) and macrophages leading to impaired production of IFNs and ILs. It may be noted here that macrophages form the major cell component of the alveolar space [21].

5. Decline in production of new naive lymphocytes and long-term immunoglobulin producing B lymphocytes [17].

6. T-cell functional dysregulation [22].

7. Increased secretion of pro-inflammatory cytokines by secretory phenotype, a process known as “Inflamm-ageing” [21]. It is caused by accumulation of memory and effector cells [23].

Many of these effects of ageing on the immune system are considered to be significant factors in pathogenesis of severe cases of Covid 19 (1,24). Most of these immune dysfunctions of IS are improved by gold nanoparticles, as we shall see in the following section (Figure 2).

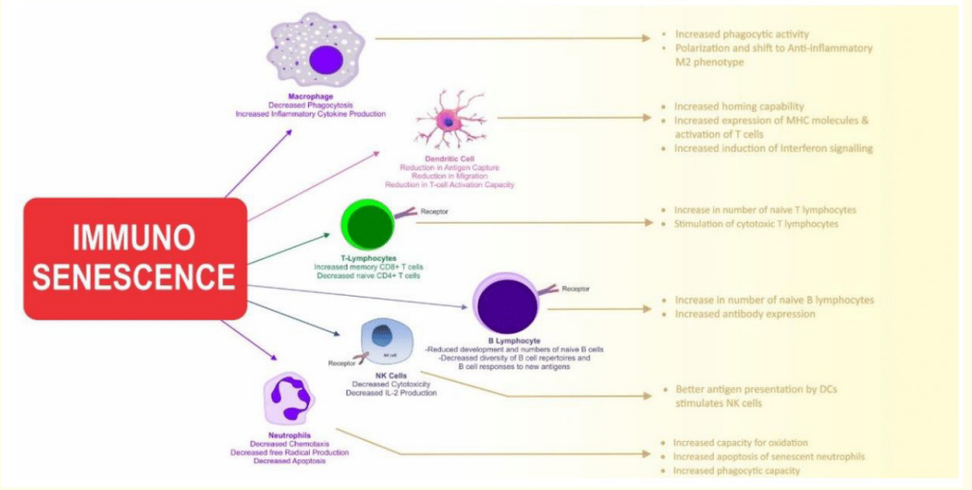

Figure 2: *Effects of Immuno-senescence on different immune cells and remediation by Gold nano Particles.*

Effects of gold nanoparticles on Immune cells

Gold nano-particles (GNP) can vary in size from 3 nm to 80 nm and particles can also coalesce to form agglomerates. Different sized GNP are found to have different effects on the immune cells as regards their uptake and functional responses. More importantly, GNPs functionalized with peptides or oligonucleotides have much greater effects as compared to bare particles [25]. Surface modification improves their stability and biocompatibility; they can be modified with compounds carrying functional groups, such as cyano (-CN), thiol (-SH), carboxy (COOH) and amino ($-NH_2$) groups, known for their high affinity for gold. Additives having these functional groups can be used as capping agents for gold [26].

Gold nanoparticles have been found to have significant effects on all components of the immune system. A brief summary of the effect of GNP on immune cells is given below.

Effects on stem cells

- Administration of GNP has been found to increase total WBC count and haemoglobin content. This indicates that GNP are able to induce the hematopoietic system [4].
- The internalized GNP may interact with proteins located in the cytoplasm or provide mechanical stimuli which trigger off a chain of biological alterations and modulate cell behaviour. Increasing evidence is suggestive of an inherent ability of GNP to promote stem cell differentiation [27].
- GNPs modulate the expression of differentiation-relevant genes that promote osteoblast differentiation from mesenchymal stem cells (MSCs) and inhibit their differentiation into adipocytes [28].

Effects on dendritic cells

The entry of the virus or bacteria into DCs has been postulated to be via two mechanisms. DCs can phagocytize apoptotic cells infected by virus, or these may be infected with virus primarily. After this, the DC home into the draining lymph nodes and present viral antigens to T cells. Nanoparticles can increase the homing capabilities of DCs, leading to faster activation of T cells [29]. Recent studies reveal that nanoparticles can affect all steps of DC induced immunity [30]. NPs including gold nano-rods favour the maturation process of DCs [31,32]. NPs have also been shown to increase the expression of MHC and co-stimulatory molecules on the DC surface. Since DC maturation and antigen presentation is crucial for induction of T cell proliferation, differentiation and activation, all of the latter can be promoted by nanoparticles [30]. In brief, the effects of GNP on DCs are:

- Uptake of GNP by DC results in increased induction of g-interferon, IL-8, IL-1b and IL-6 [33,34].
- GNP were found to promote the secretion of IL-12p70 which is directly involved in activation of T cells. The DC also showed development of long dendrites and an increase in the amount of MHC-II molecules, which present antigens to T lymphocytes [35].
- Gold NP are able to prime DC maturation, causing enhancement of cellular and humoral immune responses by synergizing the immunogenicity of antigens [30].

- Solid GNP have been found to be an efficient platform for delivering multiple immuno-stimulatory ligands to innate immune cells. This was evidenced by demonstrating potent immune-activation of bone marrow derived DCs [6].

Effects on NK cells

In a recent study, it has been demonstrated that 12 nm gold nanoparticles induced cell mediated responses accompanied by inflammatory natural killer (NK) cell stimulation [36].

Effects on macrophages

Effect on respiration and cytokine secretion: It has been demonstrated that both nonconjugated GNPs and their conjugates with high- and low-molecular-weight antigens, on entry into rat peritoneal macrophages, enhance their respiratory activity and the activity of macrophage mitochondrial enzymes. GNPs also have greatly increased the production of IL-1, IL-6 and IFN-γ [37].

Effect on shape: Exposure to GNP may also modulate the gene or protein expression of macrophages, since there is a change in morphology to a spread shape, as compared to round cells when untreated with GNP [28].

Macrophage polarization and differentiation into anti-inflammatory phenotype

Macrophages play a key role in the pathogenesis of serious illness resulting from SARS-CoV 2 infection [38-40]. It is being increasingly accepted that SCoV is unable to elicit strong induction of IFN response in human macrophages [41]. A dysregulated macrophage response, as seen in macrophage activation syndromes, is also known to occur in SARS-CoV infections [39]. Broncho-alveolar fluid (BALF) from Covid 19 patients revealed mononuclear phagocytes (MNP) to be around 80% of all cells with abundance of inflammatory monocyte-derived macrophages and a depletion of tissue-resident alveolar macrophages. The source of the large amounts of cytokines observed in Covid-19 patients is proposed to be monocytes and macrophages [42].

However, macrophages are also considered as the master immune cells in the resolution of Acute Lung Injury (ALI). In the initial stages of lung infection, M1 macrophages secrete large amounts of pro-inflammatory cytokines upon stimulation of PRRs (pattern recognition receptors). Later, in the resolution phase, macrophage phenotype shifts to the reparative M2 type, resulting in secretion of anti-inflammatory cytokines which act to subdue the inflammation and promote tissue repair. Thus, macrophage polarization and activation play a key role in initiation and resolution of ALI [43].

Mechanical and physical forces have recently been found to play a role in the polarization of macrophages [44]. Lung macrophages lining the alveoli are subjected to high amounts of stretch and mechanical loading, and these influence the differentiation of macrophages. Macrophages are known to actively ingest nanoparticles including GNP utilizing macrophage scavenger receptor A, and the larger NPs get located in the cytoplasm [45]. These large nanoparticles affect the shape and loading of the macrophages. Raimondo and Mooney found that injection of IL-4 conjugated gold nanoparticles into injured mice skeletal muscle resulted in an approximately twofold increase in the percentage of M2a macrophages and an approximately twofold decrease in M1 phenotype macrophages. Injection of soluble IL-4 did not cause these effects [46]. In a recent significant study in mice lung macrophages, it was found that peptide-coated Gold NP could induce macrophage polarization and differentiation from inflammatory M1 phenotype toward anti-inflammatory M2 phenotype, effectively regulating lung inflammation, protecting lungs from injury and promoting inflammation resolution [43].

Effects on lymph nodes and lymphocytes

Effect on lymph nodes: In a study done on rat mesenteric lymph nodes, oral administration of GNP was found to activate migration, proliferation and differentiation of lymphocytes [47].

Effect on cytotoxic T lymphocytes

- Lin., *et al*. reported that GNPs in complexes with peptides are taken up effectively by DC. A high peptide density on the GNP surface can stimulate cytotoxic T lymphocytes better than can free peptides [48].

- Similarly, GNP coated with antigens show better presentation of antigen by the MHC-1 complex to CD8+ cells resulting in increased activation of an epitope-specific immune response by cytotoxic T cells [33].

Effects on B lymphocytes

On treatment of a murine B lymphocyte cell line with GNP, an NF- kB-regulated luciferase reporter was activated, and this correlated with increased antibody expression. There is evidence to suggest that GNPs increase the production of B lymphocytes, activate B cells and enhance IgG secretion [49].

Effects on neutrophils

GNP get trapped by neutrophils in their extracellular traps (NETs) soon after administration. The NETs are composed mainly of DNA and a variety of antibacterial proteins [50]. These cell-gold networks may contribute to alerting the immune system by activating DNA receptors like TLR9 [3].

Some studies have pointed towards activation or acceleration of apoptosis of neutrophils by GNP [51]. Clearance of dead neutrophils takes place in the Bone marrow also, apart from the liver and spleen, and in this way the GNP can reach the haematopoietic stem cells [52], to improve hemopoiesis and stem cell differentiation as given in section effects on stem cells.

From the above listed effects of Gold Nano-Particles on the various cells of the immune system, it is clear that the GNP can improve, and in some cases even reverse the effects of ageing on the immune system. As we shall see later in this discussion, when GNP is administered along with ghee and raw honey (which provides numerous capping agents), these positive effects of GNP are enhanced manifold, priming both arms of the immune system to effectively defend against attack by bacteria and viruses.

Honey and its constituents as capping agents for Gold NPs

Honey is a complex mixture containing several oligosaccharides, amino acids, polypeptides and proteins [53]. All or some of these molecules can spontaneously bind to GNP, thus capping the latter and increasing the Immunogenicity (section properties of gold nanoparticles). The amino acid having significant presence in honey is Proline, which is part of the protein antigen in a large number of viruses [54]. Polyreactive natural antibodies can recognize these proline-containing structures since proline is usually present at solvent-exposed sites in proteins, like loops, turns and N-terminal first turn of the helix [55,56]. Repeated exposure to the vast array of GNP-conjugated antigenic molecules including proline, in honey can result in “trained immunity” of hemopoietic stem cells, dendritic cells, NK cells and enlarge the repertoire of T cell receptors [57].

Raw honey also contains glycoprotein peptides, which is significant because glycosylation improves the stability and immunogenicity of the peptides [58]. Peptidoglycan is a well-known PRR agonist that stimulates innate activation through Nod-like receptor 2 [59]. All of the immunoglobulins complement components and the MHC 1 and II which take part in antigen presentation are also glycosylated [58]. Several studies have indicated that glycosylation has a major influence on protein antigen uptake and proteolytic processing [60]. Thus, the glycosylated peptides in honey can act as potent stimulants of the innate immune system and also activate adaptive immunity. Since the innate immune system is antigen non-specific, activation of this system can lead to responses against a large variety of new and unknown antigens by the mechanism of trained immunity [57,61].

Ghee

Ghee is simply clarified unsalted-butter, prepared by heating butter till the translucent butter becomes transparent. Though ghee contains almost 95.5% fat, there are large number of other compounds as well including fat soluble vitamins, minerals and amino acids [62].

The milk fat globule membrane contains phospholipids like glycerophospholipids and sphingolipids, (glyco)proteins, glycolipids (i.e. cerebrosides and gangliosides), total and partial glycerides, free fatty acids and cholesterol [63]. The phospholipids in ghee act as emulsifying agents and can create liposome-like particles when used in emulsions. Lipid coating of bio-decorated GNP helps in lymphatic uptake of the particles, directly exposing the immune cells to the Particulate Antigens, since the immune cells reside in the lymphatics.

Manufacturing process of gold nano particles

Commercially available GNP

Current trend in gold NPs synthesis is the utilization of biological compounds (e.g. pomegranate, star anise) instead of chemicals (e.g. citrate), as the previous chemical synthesis method utilized harmful chemicals that have been shown to be toxic at cellular level [64]. This current biosynthetic approach is not only environmental-friendly but also eliminates the toxicity of gold NPs vital for medical diagnostic and therapeutic applications [65]. Depending on the conditions employed, GNP can be produced in sizes varying from 1 nm to 100 nm.

Calcined Gold (Swarna bhasma)

The process of manufacture and mode of administration is of prime importance in Ayurveda, especially in case of heavy metals. Metallic preparations of mercury, for example, can prove lethal if not prepared meticulously and as per the instructions given. Gold also has to undergo a specific time consuming process in order to be able to impart its proper benefits [66]. It is for this reason that there are differences in the therapeutic efficacies of chemically produced GNP and traditionally processed nano gold. Swarna bhasma (SB) preparation employs sulphur, castor leaf and aloe Vera juice, using earthen pots. In a study carried out on mice to evaluate the effects of SB and Auranofin (an antirheumatic agent), the former was found to increase the count of peritoneal macrophages and the phagocytic index, while the latter had a suppressive action on these parameters [67].

Swarn Prashan (Gold sup) -- The Oral GNP Formulation

Traditional use

Gold is known as Swarna in India. Though gold is part of the formulation in several preparations of Indian medicine, the two formulations with parallelogy to GNP are calcined Gold ash (bhasma) and SwarnPrashan (SP). Calcined gold ash is traditionally used for tuberculosis, anaemia, cough, sterility and muscular dystrophy. It is also used for its anxiolytic, anti-depressant and restorative actions. Studies have also shown effects on immunity; one study in mice showed increased counts of peritoneal macrophages with greater phagocytic index [68].

SP has been recommended in Ayurveda for neonates and infants, to be started soon after birth [9]. Perhaps the persisting and pervasive tradition in India of giving the new-born some honey has its genesis in the original prescription of gold sup. The cost and inaccessibility of pure gold for the impoverished Indian masses must have led them to a cheaper alternative of simple raw honey, leaving the original formulation of Honey-gold for the nobility and the rich merchants.

Botulism

The recurring problem with raw honey is that of Botulism. The origin of Clostridial contamination of raw honey is still under conjecture, but it is probably a result of the harvesting method wherein the bee-hive is toppled from its high perch on a tree or cliff, only to fall upon the dusty ground [69]. The hive is then picked up and the honey extracted by manual squeezing. This problem can be easily tackled by placing a clean sheet below the trajectory of fall of the hive; and other simple hygienic measures like cleaning hands before squeezing and putting the hives and honey into clean containers. In any case, clostridial contamination only rarely causes problems in adults.

The formulation

Swarn Prashan (SP) is made simply by mixing gold ash with honey and ghee (clarified butter). The only rule to be followed is to use honey and ghee in unequal proportions by weight.

The formulation of Swarn Prashan can then be described as below:

- Ingredients: Gold ash, raw honey, ghee.
- Amounts used per dose for adults: 25 mg gold ash, 2 gm honey, 8 gm ghee. Conversely, it can be: 25 mg gold ash, 2 gm ghee, 8 gm honey.
- One dose of 10 gm of SP would be two tea-spoons full.

The important point is that honey and ghee are not to be used in equal proportions by weight. The reasons for this are a subject of another paper. Another important point is to thoroughly stir or whip the mixture before use, so as to create a homogeneous ok suspension. Also, vigorous mixing will ensure separation of agglomerated gold particles, as also their conjugation with the various amino acids, peptides and saccharides in honey.

For the specific purpose of using gold sup as a vaccine against flu and Covid-19, the author also recommends adding of extract of *Tinospora cordifolia. T cordifolia* extracts are available as generic "Giloy sat", which is a powdered dry extract, or the distilled essential oil may be used, in amounts of 500 mg to 1 gm per dose.

Tinospora cordifolia

T. cordifolia (TC) is a well studied herb having a myriad of biologically active compounds, and medicinal properties like anti-oxidant, anti-diabetic, anti-inflammatory, anti-microbial, hepatoprotective, immunomodulatory and anti-neoplastic activities [70]. Large number of studies support the immunomodulatory effects of TC. More and Pai found the extract of TC increased the production of reactive oxygen species in human Neutrophils, boosted the phagocytic activity of macrophages and enhanced nitric oxide production [71]. It was also found to increase cytokine production and stimulate immune effector cells [72]. TC extracts also promote B cell differentiation and activate cytotoxic T cells [73]. Significantly, an immunomodulatory protein from stem of TC was found to be a strong immunogen by itself and also worked as a powerful adjuvant [74].

Rationale of gold sup (Swarn Prashan)

The poor response to vaccination including Influenza vaccines in the elderly is presumed to be due to IS. The various strategies to enhance vaccine effectiveness in the aged include mucosal route of delivery, use of adjuvants and using increased amounts of antigen [75].

All of these strategies find their resonance in the oral preparation of HGG (honey, gold, ghee). The presence of a vast array of antigenic molecules in honey, including carbohydrates, proteins and lipids, keeps the immune cells and the entire immune system in prime condition to respond to a multitude of new micro-organisms that it may encounter. Gold NP acts as a powerful adjuvant [15] and absorption through the mucosa of the upper GIT ensures the introduction of the vaccine components to the huge and spread out mass of lymphatic tissue in the gut. The adjuvant- potentiated hydrophobic vaccine is avidly taken up by the gut macrophages, mucosal M cells and DCs, to be presented to the lymphocytes in the Peyer's patches, stimulating both the innate and adaptive immunity.

Further, it has been established that Multivalent antigens, that is, antigen molecules with more than one identical epitope per molecule promote B-cell receptor (BCR) cross-linking and facilitate internalization of antigen [76]. In a study on antigen coated polymer particles, the particles (particulated antigens) clustered unbound BCRs and contributed to enhanced intracellular signalling and elicited antibody

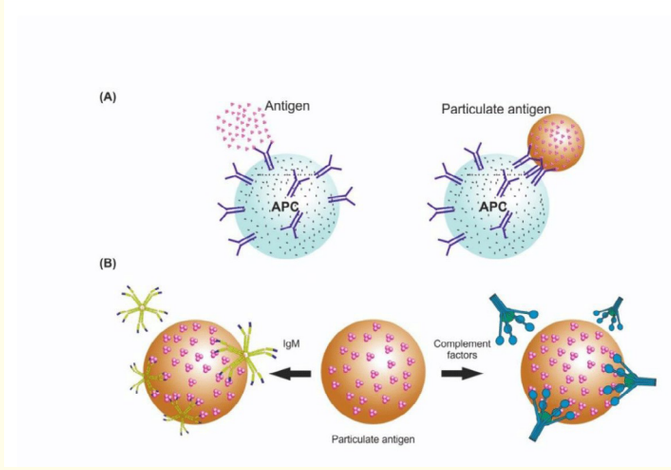

Figure 3: *(A) shows how multivalent interactions by nanoparticle vaccines promote B cell receptor clustering and facilitate receptor-mediated internalization. (B) illustrates enhanced binding of multimeric immune factors, such as IgM and complement factors, to nanoparticle vaccines*

production; in contrast, free antigens failed to trigger humoral responses [77]. Highly repetitive surfaces are also known to bind strongly to natural IgM antibodies through multivalent, high-avidity interactions [78]. Macrophage and DC uptake of particles is also facilitated by IgM antibody binding and this in turn can enhance immune processing through increased antigen presentation [79] (Figure 3).

Now, thiol and amine groups on peptides cause bioconjugation with GNP. GNP on coming into contact with the thiol group on a cysteine amino acid, can spontaneously form strong covalent linkage. This coordinate covalent bond forms because the strong interaction between sulfur and gold drives the sulfur atom to fill the free orbitals of a gold atom [80,81]. Amine groups can also form bonds using amides. Since raw honey contains large numbers of plant and animal peptides and amino acids (though in relatively small amount), such bonding will invariably occur when honey is thoroughly mixed with GNP. Since GNP have large surface area and consequently high surface energy, physical adsorption will also take place spontaneously [82]. The formation of protein corona over GNP is a well known phenomenon [83].

Ghee is a part of the formulation of SP. Ghee is important because it acts as a carrier and along with honey, forms a thick suspension which coats the conjugated gold particles. The phospholipid content and other emulsifying agents in ghee can form liposome like particle encasing the gold-antigen particles. Hydrophobic nanoparticles or particles with lipophilic material have been shown to be preferentially taken up by macrophages in comparison to their hydrophilic counterparts [84]. Thus, the gut mucosal macrophages and DCs will have more avidity for the GNP-honey-ghee (HGG) particles, leading to better absorption. Also, in association with lipid, the GNP particles can be absorbed by the lacteals, and then through the mesenteric lymphatics into the thoracic duct, from where they will reach the great veins of the neck [8]. The liver is thus bypassed, preventing the bulk of the GNP from being taken up by the Kupffer cells in the liver sinusoids. Thereby, antigen-GNP particles can reach the lungs and exert their action on the lung macrophages.

Action of bio-decorated GNP as synthetic nanoparticle vaccine

Synthetic nanoparticles have been increasingly used for vaccine design [85,86]. With particle sizes varying between 25 and 250 nm in diameter, these viral sized nanoparticles, including GNP have been shown to be capable of inducing strong humoral and cellular immune responses against antigen targets. This is attributed to GNP having several viral mimicry features like similar size, multivalent antigen

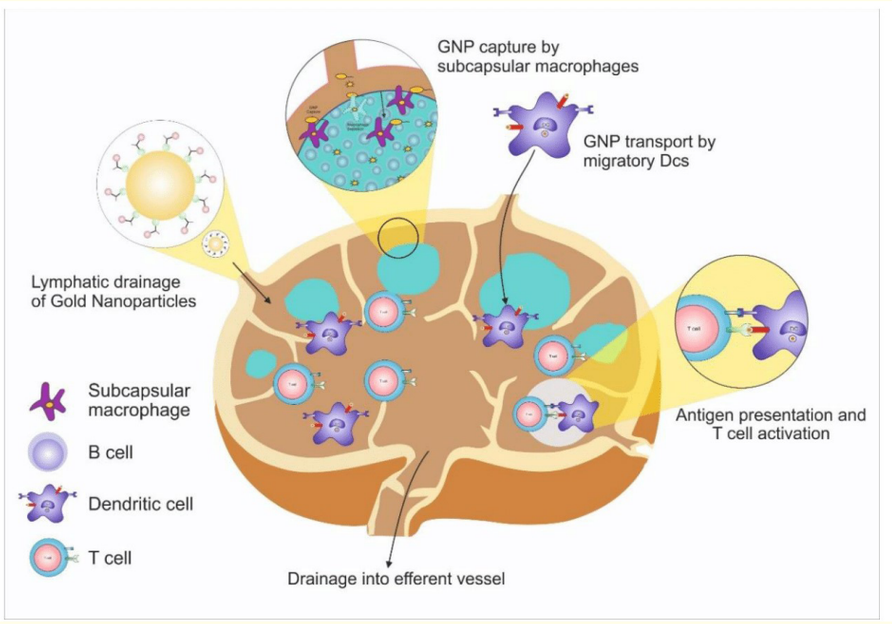

Figure 4: *Showing advantage of lymph node delivery of GNP. Gold NP that enter lymph nodes through interstitial lymphatic drainage can be captured by LN resident macrophages. GNP ingested by mucosal DCs in gut lumen will be carried to the LNs by migration of the DCs. Enhanced delivery of bio-decorated GNP facilitates antigen presentation and T cell activation.*

display and adjuvanticity [79]. Since GNP can be functionalized with hundreds of antigens, they can emulate the antigen display on viral particles [87,88]. Such mimicry of repetitive pathogen surfaces enhances antibody responses by cross-linking B cell receptors (BCR), activating complement and facilitating antibody binding. Antigen coated GNP can also enhance lymph node delivery by as much as six times, as was demonstrated when coronavirus spike proteins were delivered with 100 nm GNP [82] (Figure 4).

Immunopathology of SARS-CoV-2 and the role of Honey-Gold-Ghee formulation in conferring protection

Pathogenesis

The pathogenesis of SARS-CoV 2 infection is complex, and large gaps remain in the understanding of the mechanisms causing the cytokine storm. Though the majority of those who get infected recover, yet some succumb, primarily as a result of uncontrolled inflammation in the lungs. In the severe cases, some immunological features have been clearly identified. These are:

- There is a defective immune response which causes a pro-inflammatory feedback loop that results in accumulation of immune cells in the lung [89].
- The SARS- CoV 2 virus antagonizes interferon responses [90]. Antagonism occurs at various stages of the interferon signalling pathway, including by preventing PRR recognition and by inhibiting downstream interferon signalling.
- Lymphopenia is a hallmark, particularly reduction in T cells [91,92].
- There is depletion and exhaustion of NK cells [93].
- Interference with the differentiation and function of DCs, hindering subsequent adaptive immune responses [94]. Since SARS-CoV 2 infects DC, defective function of DC might lead to T cell apoptosis and exhaustion and contribute to the immunopathology of COVID-19 [95].
- Depletion of alveolar macrophages and proliferation of inflammatory monocyte-derived macrophages, leading to hyperinflammation and cytokine storm [39].
- T cell exhaustion [92]--T cells from COVID-19 patients had significantly higher levels of the exhausted marker PD-1 [42].
- Increased apoptosis of neutrophils in the lung parenchyma.

Immune aspects

While the adaptive immune system is considered important in the reparative phase of lung inflammation, it is the innate immune system that is of more significance in preventing the virus from gaining a foothold in the epithelial tissues of the respiratory tract [96]. Efficient induction of the Anti-viral state is key to warding off the attack by the SARS-CoV 2 virus and is the primary reason why most of the infected populace are either asymptomatic or exhibit only mild symptoms [5]. Prompetchara and Ketloy, in their elegant analysis also subscribe to the view that innate immune responses are critical factors in determining the outcome in SARS-CoV 2 infections [97]. Thus, priming of the innate immunity is key to fight the present epidemic, and can be achieved by the Honey-ghee-GNP(HGG) formulation. It needs be stated here that use of oral MGG is to be continued over several weeks, unlike one time parenteral vaccinations. Let us now examine the dynamics of the vaccine-like effects of MGG.

Priming of innate immunity

Pathogen associated molecular patterns (PAMPs)

Epithelial cells, alveolar macrophages and dendritic cells (DCs) are three main components for innate immunity in the airway. The effects of GNP on alveolar macrophage polarization towards anti-inflammatory phenotype have already been discussed in section *Effects on macrophages*. DCs reside underneath the epithelium while macrophages are located at the apical side of the epithelium. These innate immune cells are key in the fight against viruses till adaptive immunity is established. Any invading pathogen is recognized because of the PAMPs located on their surfaces. Toll-like receptors (TLRs) present on cell membranes of immune cells recognize these PAMPs which may include lipids, lipoproteins, proteins, polysaccharides and nucleic acids of bacterial, viral, parasite, and fungal origins. The recognition of PAMPs by TLRs occurs not just on cell membranes, but also in endosomes, lysosomes, and other locations in cells [94].

Macrophage and NK cell functions

The intestinal mucosa harbours the largest number of macrophages in the human body, and these will take up the biodecorated GNP particles, migrate to the lymphatic tissues and present to T cells [98]. As noted above, the Coronavirus 2 impairs the recognition of PAMPs, preventing interferon signalling. HGG use over a period of time can improve PAMP recognition by the mechanism of trained immunity [99]. Honey contains several glycoproteins [53], besides polysaccharides, proteins and nucleic acids. Glycoproteins in honey are of special significance, since glycosylation of peptides magnifies the antigenicity several fold [58]. The TLRs on macrophages in intestinal mucosa recognize the antigenic PAMP-like molecular configurations in honey, and take them up, stimulating the T cells, including NK cells. Moreover, stimulation of TLRs by peptide-associated gold NP (particulated antigen) is much more than by peptide alone [79]. Repeated exposure to the large assortment of immunogenic stimuli lends to better recognition of PAMPs by the PRRs on mononuclear phagocytes (trained immunity), with more efficient IF-g responses and downstream signalling. Furthermore, the primed functional state of trained cells appears to last long, and it can provide protection from causative agents that comes into contact with the host during this period of time [100].

Trained Immunity

Trained immunity (TI) is considered by many to be the reason for the less severe Covid-19 disease seen in children [101]. This recently coined term signifies the adaptive characteristics of the innate immune system, underscored by its ability to construct immunological memory and respond in a nonspecific sensitized manner to reinfection [102]. Trained innate immunity has been found to reside in monocytes, macrophages and NK cells. Clues to this phenomenon were found when after BCG immunization, trained NK cells showed protective effects towards Candida albicans infections after one week [103]. Subsequently, several antigens have been discovered that can confer trained immunity, including measles, vaccinia and oral polio vaccines, b-glucan and Plasmodium falciparum [104].

The establishment of innate immune memory relies on epigenetic modifications resulting from DNA and histone methylation [105]. The persistence of epigenetic modifications permits cells to remain in a ‘‘trained’’ functional state and allows increased accessibility to proinflammatory genes, facilitating faster and increased responsiveness after re-challenge. This process also takes place at the level of myeloid bone marrow progenitors, leading to a myelopoiesis bias and release of monocytes with a heightened preparedness to respond to pathogens [106]. Such trained monocytes, after release from bone marrow, will circulate all through the body including the lungs.

Importantly, a second level at which trained immunity is induced is represented by the local environments in the tissues. Experimental

studies have demonstrated that alveolar or lung macrophages can also undergo long-term reprogramming after infection [107]. Since the non-specific cross-protection offered by TI enhances immune responses during subsequent infections by same or different microorganisms, this discovery has recently led to development of trained-Immunity based Vaccines [100]. In fact, Netea has proposed development of vaccines based on this concept against SARS-CoV 2 [99].

Dendritic cell function

Dendritic cells avidly take up glycan structures and polysaccharides [108,109]. The glycans and sugars in honey, bound to GNP, on being avidly taken up by the DCs in intestinal mucosa will then be rapidly presented to T cells in Peyer's patches and mesenteric lymph nodes, provoking a sharp immune response. In Covid-19 patients, MHC class I and II upregulation was found missing [110] indicating that DC function was impaired. Since NK cells are important for attacking the virus, insufficient expression and presentation of viral antigens by DCs to NK cells leads to decreased activation of the latter. The absolute numbers of NK cells and cytotoxic CD 8+ cells have also been found to be reduced in patients exhibiting mild and severe disease. There is also clear evidence of exhaustion of these two groups of mononuclear cells [93]. As given in section effects on dendritic cells, GNP can improve expression of MHC on DCs, activate CD 8+ cells and cause NK cell stimulation, leading to more efficient viral elimination.

Lymphopenia

GNP have been shown to cause lymphocyte proliferation, with increased number of naive B lymphocytes [47] Greater variety and numbers of natural antibodies produced by bone marrow lymphoid progenitor cells allows for an early and efficient humoral response to the invading virus [49].

Senescent neutrophils cause large secretion of cytokines

This immense secretion of cytokines contributes to the cytokine storm. GNP can cause apoptosis of senescent neutrophils and increase infusion of fresh neutrophils into the circulation. Since there is a bias towards myeloid progenitor stem cells in the bone marrow, increased apoptosis of senescent neutrophils does not cause any decline in total number of circulating Neutrophils. Diminished apoptosis of Neutrophils in the lungs would also contribute towards decreasing the huge surge in cytokine.

Thus, we can see that MGG has the potential to modulate the immune system at several levels, especially in priming the innate immunity. Though administered orally, the effects on the immune cells, including macrophages and DCs would be systemic, since these APCs are highly mobile and travel within the lymphatic system. The increasing evidence pointing towards the "training" of the hemopoietic stem cells leads to global immune activation and protection from microbial invasion of all sorts, including viruses. In fact, Mihai Netea has proposed using Trained Immunity as a tool for reducing susceptibility to and the severity of SARS-CoV-2 infection [99].

Discussion

SARS - Coronavirus 2 infection results in a range of effects, from mostly asymptomatic and mild cases to more severe manifestations. The more severe disease and mortality in the aged population readily lends credence to the proposition that immuno-senescence related changes in the immune system make the elderly more susceptible to severe form of the illness. Gold NP can play a significant role in reversing several of the disorders related to IS. Given along with raw honey and ghee, even further priming of the innate immunity can take place through trained immunity. Powerful immunomodulatory herbal extracts added to MGG mixture can further enhance the immunoprotective effects, offering a simple and potent modality to combat the pandemic ravaging lives and economies all across the globe.

Conclusion

The concept of using Swarn-prashan as a vaccine against viral and microbial attack looks simple, yet there are some difficulties that have to be overcome. The primary problem is the acquisition of safe honey, meaning thereby raw honey that is harvested hygienically, tested for presence dangerous microbes, and checked for peptide and enzyme content. The second, and readily surmountable hitch, is the identification of biologically safe and immunologically active Gold NP. The author believes these two straight-forward obstacles towards successful implementation of the instantly available Honey-Ghee -Gold NP vaccine, can be managed efficiently by routine laboratory methods. Even more conveniently, the HGGNP formulation can be made at home by using the pure gold ash and honey available in the market. Even though the immunological effects may not be optimum as those obtained with raw honey and pure ghee, still they should be enough to offer significant protection. It is not always that very complex procedures and processes yield valuable results, sometimes simplicity can win the day. Leonardo da Vinci, the perfectionist who is acknowledged by many academics to be the greatest genius of all time, and whose complexity and intricacy of paint work is still being studied today, had this to say about it: Simplicity is the Ultimate Sophistication.

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Volume 7 Issue 8 August 2020