Chapter 22

Gastrointestinal Immunoregulation and the Challenges of Nanotechnology in Foods

MaryAnn Principato

Additional information is available at the end of the chapter

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1. Introduction

1.1. Nanoparticles: Physicochemical characteristics and applications in foods

Nanoparticles are elemental three dimensional structures that are typically between 1-100 nanometers (nm) in size that exhibit unique physiochemical characteristics that provide the basis for their utilization, and present unique challenges associated with the development of new applications [1, 2]. Because of their size, nanoparticles provide the opportunity to interact with human physiology at the subcellular level, affording many potential uses in nutrient and drug delivery, vaccination therapies, and tissue repair. Specific physiologic applications can be achieved by chemical modification of the nanoparticle to achieve increased blood circulation parameters thus increasing their residence time in the tissues, or by the specific targeting of tissues using ligands. The uses of nanotechnology in foods are as complex and varied as the types of formulations that can be created with this technology. Current technological applications that impact foods include the manufacture of food packaging, including packaging that incorporates antimicrobial agents such as silver, [2] or detection particles (gold), flavor enhancement, and delivery of dietary supplements and nutraceuticals [2-5]. While their potential or actual application present strong advantages, it is imperative that there be a thorough understanding regarding the physiology of nanoparticle absorption, or the consequences of their containment or integration within the mammalian physiological and cellular environment.

1.2. Food packaging

Historically, food packaging has typically consisted of conventional materials such as paper or metal-based materials. The use of polymeric formulations improved the ability to retain

moisture and provided a gas barrier, thus extending food shelf life. Typically, the Food and Drug Administration requires that the manufacturer of food contact material comply with the regulatory requirements for each individual substance that comprises the entire formulation of the food contact material [6]. These food contact materials have typically included paper, metallic-based items, and polymeric compounds such as polyethylene terephthalate (PET), polypropylene, polyethylene, polystyrene, and others. Recently the formulation of nanocomposites has improved the ability to produce food contact surfaces that are superior with respect to their heating and gas barrier resistance characteristics [2, 7, 8]. Typically, a combination of previously approved compounds and nano-material has been used for the construction of the newer nanocomposite materials that strive to enhance the storage and preservation of foods. Nanocomposites are described as a combination of inorganic nanomaterial and a continuous phase consisting of synthetic polymers [9]. Nanoclay composites consist of magnesium aluminum silicate nanoparticles (bentonite or montmorillonite), and have proven to be a superior gas barrier for the preservation of foods [7]. The production of sustainable, biodegradable polylactide (PLA)-based polymers present the potential to reduce the dependence upon petrochemical based polymers by using alternative renewable sources to produce packaging materials with qualities comparable to presently used products. The combination of PLA with montmorillonite (MMT) nanocomposite [10-12] has been reported to produce a short term packaging material with good O_2 gas permeability, and can be converted into CO_2 and H_2O through decomposition by microorganisms [10].

Silver, which has long been recognized for its antimicrobial characteristics [13], has been among the inorganic constituents incorporated into nanocomposite materials. Prior to the advent of nanotechnology, silver had long been used as an ingredient within dental composite material [14], integrated into wound dressings [15-17] and other medical devices approved by the FDA, and is recognized as a biocide by the EPA [18]. Analysis of the antimicrobial effects of silver ion on gram positive and gram negative cell walled microorganisms demonstrated similar effects [19, 20]. Exposure of microbial organisms to silver results in the retraction of the cytoplasm from the cell wall, condensation of the DNA into electron-dense granules, and there is an accumulation of silver ions into the cytoplasm. The damage, as inferred in these studies, is due to the inability to replicate at the DNA level [19]. Additional denaturant effects attributed to the silver ion include its ability to attach to sulfhydryl groups, amino groups, and the terminal phosphate and carboxyl groups of bacterial proteins [13], essentially inactivating the enzymes involved with electron transport and metabolism. Of the electron transfer functions, cytochrome reductase and cytochrome oxidase are targeted [21]. While interest in silver's use as an antimicrobial has increased due to the observed rise in hospital and community-acquired antibiotic resistances, it is important to note that a growing microbial resistance to silver has also been reported [22]. Antimicrobial properties are similarly attributed to silver (Ag) nanoparticles [20, 23-27], and this property has spurred the inclusion of this material into a wide array of products within the foods sector including packaging and service containers, and bottles, or used as a measure to prevent or control surface contamination by *Escherichia coli* and *Staphylococcus aureus* [28]. Indeed, the incorporation of silver into MMT composite preparations was shown to inhibit the

growth of *Escherichia coli* 0157:H7, *Staphylococcus aureus*, and *Klebsiella pneumonia* on agar at levels that were 35% of the levels achieved by cefotaxime and chloramphenicol [29].

1.3. Dietary supplements and nutraceutical delivery

The encapsulation of dietary vitamins and other nutritional supplements as a nanoparticle has gained considerable interest as a means to increase the shelf life of such materials, and to improve delivery and release within the body. The engineered particles provide potential strategies with which to overcome the impermeability of the mucosal epithelium, and offer a possible means of circumventing the degradation of the nutrient by harsh degradative gastrointestinal conditions. Several candidate materials, used successfully for the delivery of drugs and vaccines, have been examined for their ability to encapsulate nutrients. Finally, compounds such as polysaccharides and proteins that are already in use within commercial food applications are attractive candidates for the production of new nanocomposite packaging and encapsulation material, as several are generally regarded as safe and are biodegradable.

Poly (D,L)-lactic co glycolic acid (PLGA) nanoparticles are widely used for the encapsulation and delivery of drugs due to their reported biocompatibility and lack of overt toxicity. The physicochemical properties of the PLGA particles are affected by specific formulation and processing parameters, such as drug and polymer concentration, solvent volume, polymer molecular weight, the type of emulsifier used in the processing and its concentration, and the aqueous-to-organic phase ratio [30, 31]. Thus, PLGA nanoparticles have been shown to adequately encapsulate hydrophobic and hydrophilic molecules albeit the latter present some challenges with respect to a lowered load efficiency, and many PLGA-encapsulated delivery systems have been designed for a wide variety of macromolecules including drugs, biologically active cytokines, and peptides [31-33].

Chitosan, an N-deacetylated derivative of chitin, has been analyzed for use in nutrient delivery due to its wide acceptance in drug delivery, and is generally regarded as non-toxic and biocompatible. Chitosan ((1 ▪ 4)-2-amino-2-deoxy-β-D-glucan) is a naturally occurring cationic polysaccharide found in the shells of shrimp, lobsters, and crab that has an intrinsic ability to bind mucin. The bioadhesive property of chitosan permits organ-specific delivery, and surface modification of the polysaccharide particle has been successfully used to alter organ delivery [34]. Chitosan has been demonstrated to induce increased permeability in Caco-2 monolayers across tight junctions as measured by changes in the measured transepithelial electrical resistance and in a ^{14}C-mannose absorption assay [35, 36]. The improved absorption across cell layers due to the opening of tight junctions is thought to be the result of ionic interactions between the cell membrane and chitosan polysaccharide. While these characteristics favor the polysaccharide's use as a delivery method for a variety of compounds, it is necessary to incorporate anionic alginate to prevent burst release of the encapsulated material due to protonation in an acidic environment. The results obtained by encapsulation of Vitamin A within dual layered chitosan-alginate nanospheres have been reported to be successful [37, 38]. In this instance, a high encapsulation efficiency and improved storage stability was achieved using double-layered microcapsules that incorporated

chitosan, alginate, calcium chloride and Tween 20. The production of combined Chitosan/ PLGA spherical particles have also been reported for the encapsulation of Vitamin A [38].With this construct, the microspheres (averaged 283 nm) demonstrated stability within an acidic environment and a lowered release rate into the gastric environment when compared to particles composed solely of PLGA. Thus, release of the target nutrient would be mainly in the small intestine where the vitamin would be absorbed. Interestingly, the material was visualized in the intestinal villi, and in the endothelium of rabbit GI.

Whey protein, derived from dairy, is recognized for its natural ability to form films and gels [7]. Whey nanospheres containing alginate have demonstrated the controlled release of an encapsulated nutrient, riboflavin, when tested in simulated gastric juices [39]. In this instance, 94 nm whey nanoparticles were constructed using an emulsification and cold gelation method, which averts the use of toxic solvents, and modification of the alginate concentration provides some control over degradation of the particle by pepsin in their assay. The encapsulation of viable probiotic yeast cells has been reported using whey –alginate microspheres produced by a cold gelation extrusion technique [40]. The encapsulation of a hydrophobic, fat-soluble nutrient can be achieved using casein maltodextrin nanoparticles produced by the Maillard reaction. In this reaction, the ε-amine groups found on the protein's lysine residues are covalently bonded to the aldehyde of reducing sugars. Particles produced in this manner consist of an exterior composed by the bulky hydrophilic domains of casein. The result of this design is a particle with increased curvature, i.e., a smaller diameter, containing an outermost saccharide layer and a hydrophobic inner core. Once the optimal casein: maltodextrin ratios were determined for the formation of the conjugates, incorporation of oil-soluble vitamin D resulted in particles that were 30 nm in diameter and demonstrated significant protection of the vitamin at low pH values that simulated gastric juices [4].

Liposomes composed of polar lipids such as lecithin have been used as delivery systems for antimicrobials, colors, and antioxidants. However, best results have been reported incorporating an additional layer of material such as the cationic polysaccharide, chitosan. Liposomes composed of soy lecithin and prepared by homogenization, and combined with chitosan with stirring and sonication, were used to encapsulate grape seed extract [3]. In this instance, the particle size increased with the addition of the grape seed extract due to surface incorporation of the grape seed extract into the liposomes' layer. This was rectified by production of particles containing multiple polymer layers composed of chitosan and citrus pectin; grape seed polyphenols were no longer exposed to the matrix. Finally, microspheres with a mineral composition have also been developed for the encapsulation of nutrients. In this case, the encapsulation of water soluble polyphenols extracted from green tea has been accomplished using calcium carbonate salt solutions containing phosphate and carbonate[41].

2. The Gut-Associated Lymphoid Tissue

The proposed and anticipated uses of orally-delivered nanoparticles, the use of nanoparticles on food-contact surfaces, and the introduction of microencapsulated nutrients, necessi-

tate an understanding of the events within the mucosal immune compartment known as the Gut-Associated Lymphoid Tissue (GALT), which is critically involved in the formation and maintenance of oral tolerance to introduced nutrient-derived antigens, and the generation of mucosal immune responsiveness to ingested pathogens and their toxins. The gastrointestinal tract is responsible for the digestion and absorption of ingested nutrients. This function is aided by the intestine's mucosal lining, whose absorptive surface is greatly increased by villi which project into the lumen and are composed of a single layer of epithelial cells and a rich network of capillaries and lymphatics. While the gastrointestinal tract is responsible for the absorption of nutrients, it is also the site of ongoing immune surveillance. The intestinal lumen normally contains dietary degraded products, commensal microbial flora, and any ingested contaminants including pathogenic bacteria and their products, viruses, fungi, or parasites. The resident gastrointestinal immune system must: 1) generate immunologic tolerance towards nutrients and the resident microflora, and 2) recognize and remove infectious agents and their toxins [42-44]. Oral tolerance is driven by prior administration of antigen by the oral route, generating suppressive regulatory T cells, but is also dependent upon the maintenance of an effective epithelial barrier. The role of the resident gastrointestinal $CD4^+$ T cell population for the establishment and maintenance of the tolerant state is critical [45, 46]. Investigators have reported the formation of exosome-like structures, designated as "tolerosomes, " which are assembled in and released from small intestinal epithelial cells, that seem to play a crucial role for the induction of tolerance [47]. Breakdown of oral tolerance is thought to lead to the development of food allergy and some autoimmune diseases, including inflammatory bowel diseases (Crohn's disease and ulcerative colitis) and celiac disease.

The GALT of the gastrointestinal tract consists of Peyer's patches (PP) containing B cells, dendritics, and T cells (Figure 1), appendix, draining mesenteric lymph nodes, and lymphatic follicles distributed throughout the length of the intestinal tract. The first line of immunologic defense is the provided by antibodies of the secretory IgA type found in the mucosal secretions of the gut [48]. This is supported by the observation that individuals with IgA deficiencies demonstrate circulating immune complexes to bovine and milk proteins [49]. In this case, the lack of IgA permits the entrance of food-derived antigens into the peripheral circulation, resulting in immune complex formation. The production of IgA is now known to be induced by regulatory T cells that have been activated by CD11+ dendritic cells [50]. Additionally, lymphocytes are scattered within the columnar epithelial layer (Intraepithelial lymphocytes, or IEL) and throughout the lamina propria.

2.1. T cells of the Gut-associated lymphoid tissue

Beneath the epithelial layer of the mammalian gastrointestinal tract lies a rich source of immunocompetent cells within the submucosal lymphoid follicles known as the intraepithelial lymphocytes (IEL) that comprise a significant portion of the body's T cells. The peripheral immune system contains effector T lineage cells bearing the $\alpha\beta$ T cell receptor (TCR) which are either class II-restricted $CD4^+$ T cells or class I-restricted $CD8^+$T cells. Intraepithelial cells are distinguished by the predominant presence of homodimeric $CD8\alpha\alpha^+$ T cells and T line-

age cells containing the γδ TCR [51]; interestingly, the TCRγδ lineage and TCRαβ+CD8αα populations do not retain immunologic memory of infection. However, the γδ-T cell enriched IEL function as a surveillance system for damaged or infected epithelial cells, and may modulate local immune responses by controlling cellular traffic and limiting mucosal access of inflammatory cells [52]. The γδ T cells are thought to play an important role in the pathophysiologic response to infections including Staphylococcal infection. In mice, 45% of the IEL present in the small intestine are estimated to be conventional thymus-derived lymphocytes that coexpress TCR-αβ and classical CD8-αβ. These cells primarily exhibit a cytolytic function and are recognized residents of the lamina propria, yet retain the ability to dessiminate to various anatomical sites including the gut epithelium following an antigen priming [53]. However, there are also TCR bearing αβ T cells in the lamina propria, the majority of which exhibit the activated/memory phenotype; the major histocompatibility (MHC) class II-restricted CD4+ T helper (Th) cells.

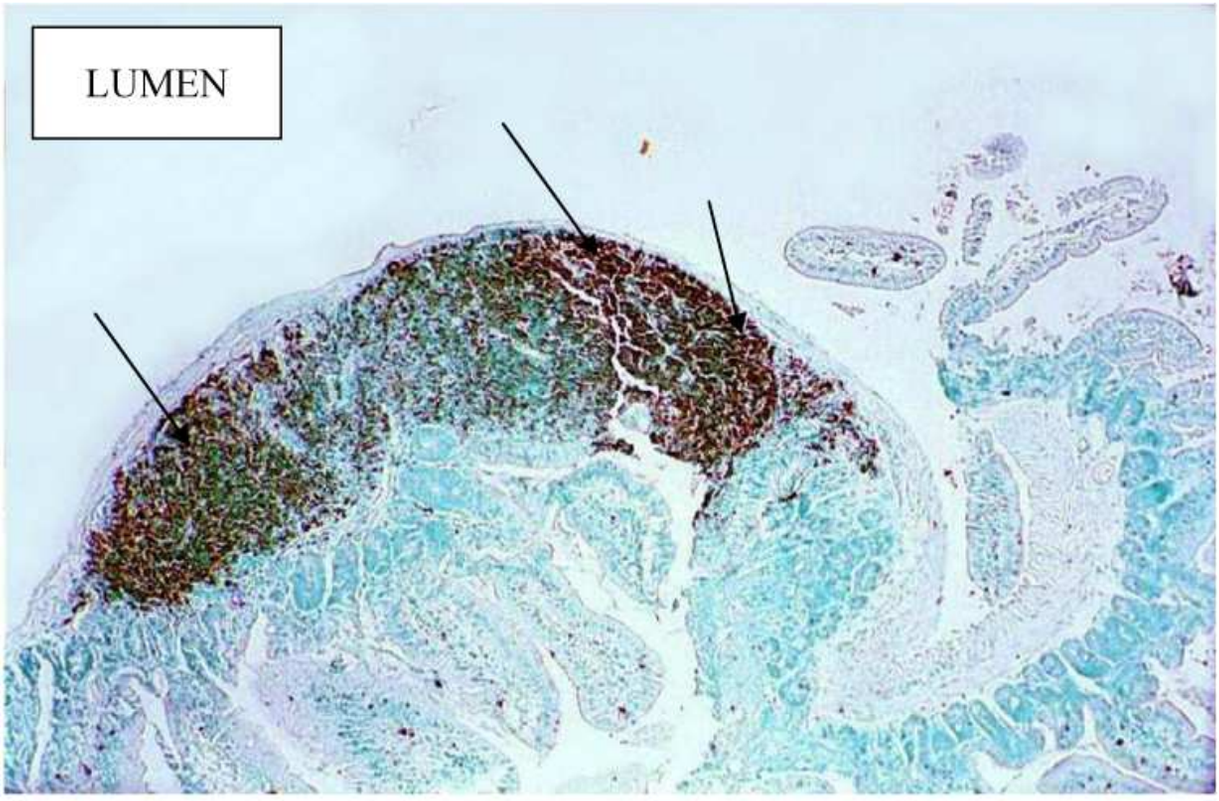

Figure 1. B220+ lymphocyte localization (indicated by arrows) in Peyer's Patch derived from normal C57Bl/10J mice. Formalin fixed tissue section [10X magnification) was stained using a monoclonal directed against B220 (RA3-6B2) and a horse-radish peroxidase conjugated antibody. B220 staining demonstrates a predominance of B cells in the unstimulated Peyer's Patch.

2.2. T cell immune activity in the GALT

During a gastrointestinal immune response, ingested antigens in the lumen enter the Peyer's Patches via the specialized epithelial cells known as M cells present in the epithelial layer

overlaying the PP. The M cells can take up particulate antigen by endocytosis and transport the antigen into the interior of the PP where the dendritic cells process the antigen and present antigen to the T cell areas of the PP and MLN, initiating T cell activation and differentiation into effector cells, that will either mediate tolerance or immunologic responsiveness [54, 55]. In experiments using genetically-defined mice, ingestion of the superantigenic food toxin, Staphylococcal entertoxin B (SEB), has been demonstrated to increase the TCR-$\alpha\beta$ populations in PP (Figure 2) such that the predominant response is generated as a result of the binding between the toxin, target T cell receptor-bearing populations containing the defined Vβ-8 sequence, and antigen presenting cells [56, 57].The result of this interaction is the receptor-mediated induction of cytokine-driven T cell proliferation, resulting in a proliferation and expansion of the SEB-reactive Vβ-8$^+$ T cells. As shown in Figure 2, normal PP contain an abundance of B220$^+$ B cells. However, the distribution of B220$^+$ B cells becomes dramatically altered following oral administration of SEB in quantities sufficient to induce illness in humans to genetically-defined C57Bl/10J mice. The B220$^+$ populations become sequestered, and the interior of the node becomes predominantly B220 negative. In this case, the PP lymph node becomes enriched for Vβ-8$^+$ T cells as determined by flow cytometric analysis (Principato, unpublished). $\gamma\delta$-T lymphocyte populations in PP, lamina propria, and epithelium have also been observed to increase following SE treatment [58].

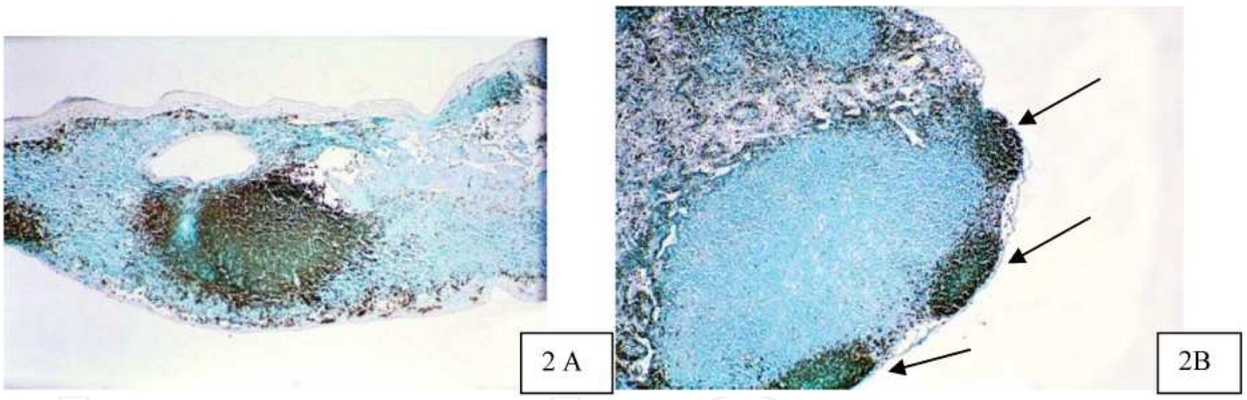

Figure 2. A. Distribution of B220$^+$ B cells in normal C57Bl/10J mice Peyer's Patch. Formalin fixed tissue section was stained using a monoclonal antibody directed against murine B220 (RA3-6B2) and a horse-radish peroxidase conjugated antibody [10X magnification). B200 staining (brown areas) demonstrates a diffuse presence of B220$^+$ B cells in the unstimulated Peyer's Patch. B. Expansion of non-B220+ (i.e., T cells) within Peyer's Patches 6 days following ingestion of Staphlyococcal enterotoxin B. A redistribution of the B220+ cells into aggregates forming below the PP epithelial capsule is indicated by arrows. [40X magnification).

2.3. T helper subsets of the GALT

The intestinal mucosa harbors all of the major T helper (Th) cell subsets (Th1, Th2, Treg (immunoregulatory), Th17) that are defined by their lineage-specific transcription factor expression, cytokine production, and immune function. The Th1 subset is critical for immune responses generated against intracellular pathogens, and provides cytokine-mediated "help" to the cytotoxic T lymphocytes. It is characterized by the production of interferon-gamma

(IFN-γ) which is controlled by the transcription factor T-bet [59]. The Th2 subset provides help for B cells and is also implicated in allergic sensitization, including those attributable to foods [60]. The specific transcription factor for Th2 cells is GATA-3, which drives the synthesis of IL-4, IL-5, and IL-13 [61]. Tregs that have arisen from antigen-specific induction of $CD4^{+}Foxp3^{+}$ T cells are critical for the induction of oral tolerance[62]. The Th17 cells express retinoic acid-related orphan receptors (RORγt and RORα) that are needed for the transcription and synthesis of IL-17 [63, 64], and provide important protection of mucosal surfaces against extracellular bacteria.

2.4. Innate immunity in the GALT

The cells of the innate immune system include macrophages, dendritic cells, and Langerhan's cells, and are involved in critical activities pertaining to the initiation and support of T cell-mediated, antigen-specific immunity. Significantly, the distribution of these cell types includes the skin and epithelia that line the internal organs including the gastrointestinal tract. Macrophages and dendritic cells are situated below the single layer of epithelial cells that lines the Peyer's Patches and lamina propria [65]. Macrophages have long been identified as components of the reticuloendothelial system and are recognized for their ability to ingest extracellular matter including proteins, cellular fragments, and debris that is foreign to the body in the process known as phagocytosis. They are widely distributed within the tissues of the body, and are crucial components of immune responsiveness and inflammation. Initial binding of the target occurs on the surface of the cell, utilizing receptors with specific capabilities. Receptors identified include surface Fc receptors that bind the Fc portion of IgG immunoglobulin, complement C3b and C3d receptors, MHC Class I and Class II, Toll like receptors (TLR), cytokine receptors, and other membrane receptors such as the C-type lectins [66] that provide additional innate functionality which supports the binding and internalization of a wide variety of targets. Opsonization of target by plasma proteins is known to improve phagocytosis, and the endocytosing vesicles have been demonstrated to consist of clathrin structures [67, 68]. Interestingly, the endosome exhibits plasticity, and its shape has been demonstrated to change depending on the material that is engulfed [69]. In an early examination of macrophage activity, Unanue and coworkers demonstrated distinct differences in macrophage effector function based on the anatomical source of the macrophage. These investigators compared the ability of alveolar and peritoneal-derived macrophages to bind and present antigen, the intracellular pathogen *Listeria monocytogenes*, to previously sensitized T cells [70]. While alveolar and peritoneal macrophages both expressed class II Ia antigen, alveolar macrophages were less efficient with respect to the uptake and presentation of antigen to sensitized T cells as compared to the peritoneal macrophages. However, opsonizing *Listeria* using an anti-Listeria antiserum to coat the bacterium enhanced the alveolar macrophages' ability to engulf the bacterium and effectively present the antigen to sensitized T cells. Once internalized, the ingested antigen undergoes intracellular metabolic and proteolytic degradation, and modification. The resulting fragment [71, 72], is transported to the surface of the cell where it is presented in conjunction with the major histocompatibility (MHC) gene molecule. This structural relationship is critical for the activation of the appropriate responding T cell, which contains a great variability

of gene sequences which must be rearranged to configure a mature, functional, TCR. This permits the specific recognition of the presented peptide sequence by the TCR of the responding T cell, and provides for the development of the adaptive immune response, which will also generate an immunologic memory of the peptide target.

Innate immunity through the Toll like receptors (TLR) is conferred with the task of recognizing a broad range of repetitive antigenic specificities that are found on a wide array of pathogens. With this type of recognition, pathogen detection is based on the ability to recognize pathogen-associated molecular patterns using evolutionarily conserved, germline encoded recognition receptors, the TLR [73-75]. Thus, while a strict sequence-dependent antigenic specificity is not required as with antigen-specific immune responsiveness, what is required is an ability to bind carbohydrate residues in a Ca^{++} dependent manner, and the recognition of conserved molecular patterns such as in bacterial cell wall components. Thus, LPS is the ligand for TLR 4, and targeted mutation of the TLR4 locus in mice results in LPS non-responsiveness [76]. TLR2 recognizes ligands found on yeast cell walls, bacterial lipoproteins [77], and lipoteichoic acid found in the cell walls of gram positive bacteria [78]; other TLR recognize bacterial DNA, or double stranded viral RNA. In humans and mice, there are now at least 10 such TLR identified. Unlike the sequence-specific receptors found on the antigen-binding T cells of the adaptive immune response, TLR are non-clonal and do not require gene rearrangement in order to become functionally mature.

Upon contact with a pathogen, the cells of the innate immune system become activated, the binding of their receptors initiating signaling cascades that turn on required transcription of target genes for the production of inflammatory cytokines, and the upregulation of costimulatory and MHC molecules necessary for the direct elimination of the infection or for the recruitment of adaptive immune responses. The binding of TLR with their target ligand induce costimulatory molecules that were first identified as the B7.1 and B7.2, or now referred to as CD80 and CD86. Thus, the responding T cell must recognize the modified target ligand which is expressed on the surface of the macrophage or dendritic cells in the context of both MHC and costimulator molecules with its sequence-specific TCR. It is clear that the cells of the innate immune system are critical to the establishment of an effective immune responsiveness against pathogens and for the recruitment of an efficient adaptive immune response. The extremely successful yellow fever vaccine, YF-17D, which induces both Th1/Th2 responses and generates powerful neutralizing antibodies in vaccine recipients, was shown to induce such a strong protective immunity as a result of its ability to stimulate multiple subsets of human dendritic cells and multiple TLRs [79]. Vaccine designs utilizing synthetic 300 nm PLGA nanoparticles containing antigen and ligands that bind TLR 4 and TLR 7 on the surface of dendritic cells, have successfully induced enhanced antigen-specific antibody responses against the immunizing antigen when injected into experimental mice[80]. The immunization protocol induced long-lived, high avidity antibody that was dependent upon the expression of the targeted TLR on both B cells and dendritics. The B cell response indicated the generation of memory-type B cells.

Macrophage and dendritic cells have been documented with respect to the striking specializations of the subsets. For instance, $CD11b^{+}$ dendritic cells of the lamina propria can sample

luminal microbes by extending their dendrites to interdigitate between neighboring intestinal epithelial cells [81], and have been reported to promote the differentiation of $Th17^{+}$ regulatory T cells following activation of TLR 5 due to exposure to bacterial flagellin [82]. Interestingly, as previously observed by Unanue and coworkers [70], anatomic localization can denote distinctions in the functional effector function within subsets of cells. Thus, CD11b+CD103+ dendritic cells of the lamina propia are found preferentially in the duodenum and rarely in the colon during the steady state, but accumulate in the lamina propria of the colon along with Th17 cells during intestinal inflammation [83]. Macrophages of the lamina propria have been demonstrated be hyporesponsive to certain inflammatory stimuli, secrete IL-10, promote the differentiation of FoxP3+ regulatory T cells [84] and are able to dampen some immune responses and intestinal inflammation. For instance, a severe dextran sulfate-associated experimental colitis can be induced in a macrophage-depleted transgenic mouse or in clodronate-treated normal C57BL/6 or Babl/c mice [85]. Finally, CD103+ dendritic cells are known to assist in the antigen specific induction of FoxP3+ T regs necessary for tolerance induction[86]. Thus, the cells of the innate immune system maintain a balance between a normal state of tolerance, and inflammatory and autoimmune responses.

3. Ingestion of nanoparticles

The ingestion of nutrients with subsequent transit throughout the lumen of the gastrointestinal tract leads to the translocation of the material across the mucosa via the M cells of the epithelial layer. M cells are specialized cells that exhibit endocytic activity, and are known to transport antigens into the interior of the PP where the dendritic cells process the antigen and present antigen to the T cell areas of the PP and MLN, initiating T cell activation and differentiation into effector cells, that will either mediate tolerance or immunologic responsiveness [54, 55]. Multiple physiochemical properties, including size and surface charge, have been shown to influence nanoparticle uptake and absorption in the gut, and the extent and rate at which the particles are removed from the circulation and their ultimate biodistribution. Thus, orally-administered non-ionic nanoparticles of 100 nm or less have demonstrated preferential absorption in the Peyer's Patch and the small intestine. Focused, engineered targeting of particles to the GALT has reported success with respect to the induction of measurable antibody responses. However, the specific immunologic mechanisms inherent to nanoparticle intake and absorption within the gastrointestinal tract have not been adequately identified, and the effector pathways that generate the immune responses measured have not been characterized.

3.1. Influence of nanoparticle size and charge

Desai and coworkers demonstrated that 100 nm nanoparticles underwent a preferential uptake in the gastrointestinal tract [87] using an *in situ* rat ileal loop model. Polylactic polyglycolic acid (PLGA) nano- and microparticles were synthesized with averaged diameters of 100 nm, 500 nm, 1 μm, and 10 μm and infused into the tissue. Tissue uptake was quantified as weight of the nanoparticles (μg) (taking into account the density of the polymer and the

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diameter of the microparticle) per square mm area of rat intestinal tissue. Infusion of the particles into gastrointestinal tissue demonstrated 100 nm particle uptake by both duodenal and ileal tissue. However, the ileum's Peyer's Patch and non-Peyer's Patch tissue demonstrated a higher uptake of 100 nm size particles. This observation was repeated using surrogate-loaded microparticles. Histologic examination of the tissue using fluorescent microscopy confirmed a greater retention of the 100 nm nanoparticles, with a concentration below the epithelial layer.

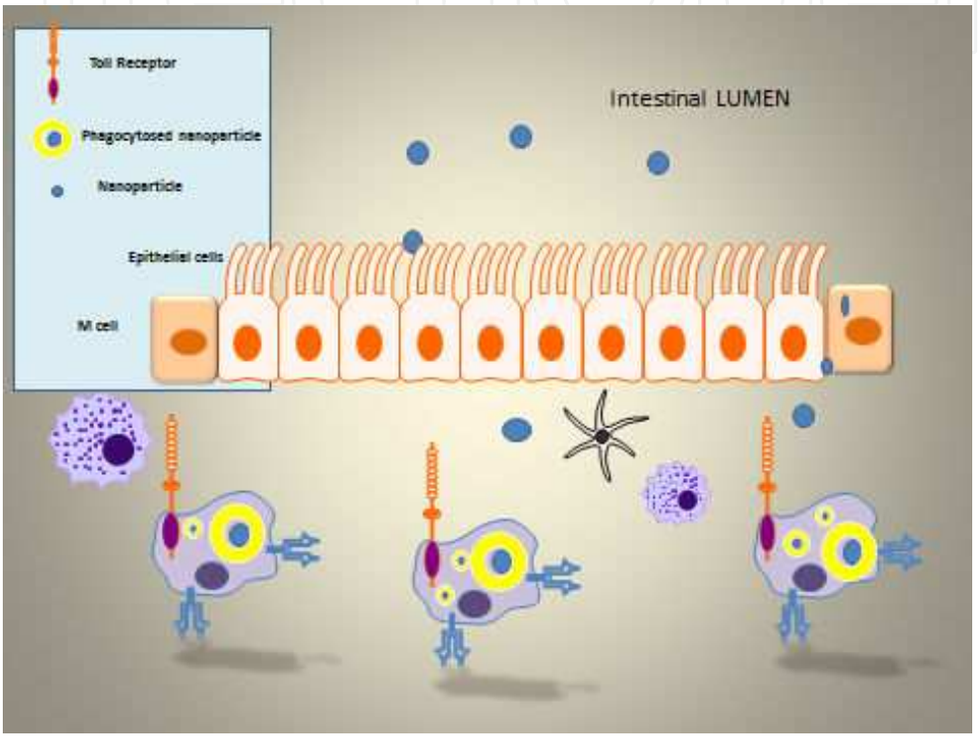

Figure 3. Schematic representation of the ingestion of nanoparticles. Macrophages and dendritic cells can be found beneath the eptihelial layer of the GALT. Ingested nonionic or targetted nanoparticles distribute preferentially below the intestinal epithelium, and can meet macrophages bearing class II and TLR molecules, and are phagocytosed by the macrophage. Actively phagocytosing macrophages are represented in the foreground; engorged macrophages are represented containing multiple particles.

The authors' observations recollect those of an earlier study utilizing latex particles [88]. In that study, Jani and coworkers conducted a 10-day feeding study in which non-ionic latex particles ranging in size from 100 nm, 500 nm, 1 micron, and 3 microns were fed to Sprague-Dawley rats. Their histologic and radiologic examination provided unequivocal evidence of a preferential tissue distribution of 100nm particles in which the Peyer's Patches, liver, and spleen demonstrated significant uptake. Significantly, their result confirmed the potential transport of particles from the gastrointestinal tract to the periphery via the lymphatics.

A separate 5 day feeding study in rats demonstrated the effects of a hydrophilic charge upon the tissue distribution of normally hydrophobic polystyrene particles [89]. In this study, commercial non-ionized polystyrene particles with a mean diameter of 60 nm were compared to similarly-sized particles coated with poloxamer 407. Their results confirmed

the earlier observations by Jani and coworkers: a preferential uptake of uncharged polystyrene was noted in the small intestines and Peyer's Patches as measured using gel permeation chromatography to quantify polystyrene in the tissue, and by microscopy. Further, a smaller concentration of particles was observed to be in the mesenteric lymphatic tissue and liver. Collectively, these data indicate a movement of the particles from the lumen of the intestinal tract to the peripheral circulation with subsequent residence in other tissues. However, charged particles demonstrated a significant reduction in uptake, 1.5%- 2% of the total administered dose of particles were absorbed as opposed to 10% uptake using uncharged particles. Interestingly, the tissue distribution was altered as a result of the poloxamer coating: the particles were particularly concentrated within the tissues of the large intestine. Taken together, these results demonstrate the importance of particle size in determining the tissue range of ingested neutrally charged particles, and the critical role of charge as a particularly strong determinant of distribution within the body.

3.2. Biodistribution

The macrophage is most often implicated in the uptake of nanoparticles and opsonization will influence nanoparticle uptake into the cells. Nevertheless, final biodistribution and disposition is likely determined by the transport of particles by phagocytic and endocytotic cells. The intraperitoneal injection of 40 nm gold nanoparticles in mice has been demonstrated to result in the localization of particles in the Kupffer cells of the liver. In this research, commercially-produced colloidal gold nanoparticles containing a negative surface charge, in sizes of either 2 nm or 40 nm, were injected either intraperitoneally (ip) or intravenously (iv) into C57Bl/6 mice, and detected within cryostat sections of liver and other organs by autometallography, which amplifies the detection of gold. Interestingly, a preferential uptake of the 40 nm particles by Kupffer cells of the liver was observed 24 hours after ip injection. Very little uptake was observed 1 hour after injection. Animals who received the particles by ip injection also demonstrated particle uptake within the walls of the small intestine, mesenteric lymph node, and in the spleen illustrating that transit of the administered particles had occurred, most likely via the phagocytic cells [90]. Using a rabbit model, orally administered chitosan/PLGA spherical particles (averaged 283 nm) for the encapsulation of Vitamin A were found along the mucosal epithelium of the lumen, and within the intestinal epithelial cells, presumably due to endocytosis. Macrophages in the lamina propia showed evidence of particles as did the endothelial cells [38].

Variations to the particle, such as addition of a polymeric coating, will alter the biodistribution. Thus, coating polystyrene 60 nm and 5.25 μm particles with poloxamer polymers will decrease uptake by liver and spleen macrophages. Importantly, increasing the thickness of the coating will alter uptake as well; in this case it has resulted in a reduction of uptake by the peritoneal macrophage [91]. Experiments in which hydrophilic negatively-charged alginate-coated chitosan nanoparticles were passively absorbed into gastrointestinal tissue demonstrated localization beneath the follicle associated epithelium of the Peyer's Patches and agglomeration of the particles intracellularly, although the specific nature of the cell was not described [92].

Significant patterns in organ compartmentalization have also been described for metallic nanoparticles.

The fate of ingested silver salt and silver nanoparticle was examined in a feeding study in which separate groups of female Wistar rats were administered 9 mg silver acetate or 12.6 mg silver nanoparticle per kg of body weight daily for 28 days. It was estimated that 63% of the daily ingested dose was excreted in the feces. The overall accumulation of either silver ion or nanoparticle was similar, and appeared greatest in the small intestine while also detectable in liver, kidney, and stomach. Autometallographic staining (AMG) detects the presence of either silver acetate or nanoparticle; thus, silver was localized to the lamina propia and submucosa in the ileum. Interestingly, silver was concentrated around the veins and portal circulation of the liver and was not preferentially taken up by the Kupffer cells of the liver as was reported with injected gold [90]. Transmission electron microscopy displayed similar localizations for both the silver nanoparticles and silver acetate; the material was found within the lysosomes of the macrophages within the lamina propia of the ileum [93].

4. Immune responses and the ingested nanoparticle/microparticle

Particulate antigens are known to induce stronger immune responsiveness to the antigen when compared to an immune response generated with the soluble form of antigen. Thus, vaccine design has recently emphasized the use of nanoparticles to maximize induction of the protective immune responsiveness. Mucosal immunizations have been viewed increasingly as an alternative to parenteral administration of vaccines, and features such as carbohydrate residue targeting by lectins has been examined by many groups [94-96]. Nevertheless, nanoparticle absorption within the GALT is still not well understood, and the effector cellular interactions involved in the generation of the induced immune response have not been fully defined.

Immunoglobulin production as a function of particle size, was measured by Gutierro and coworkers [97]. Bovine Serum Albumin (BSA) –loaded PLGA microspheres of 200 nm, 500 nm, and 1000 nm were constructed using a double emulsion technique; size was determined by laser diffractometry using a CoulterCounter® particle size analyzer. PLGA microspheres containing BSA target antigen was administered by each of three routes: subcutaneously, intranasally, or orally into 6-8 week old Balbc/J mice and the elicited immune response was measured by assaying IgG immunoglobulin production. Their results showed that IgG antibodies were elicited using each of the three sizes of microspheres when administered subcutaneously; one size did not elicit greater antibody production than the others. Further, all three sizes elicited antibody responses that were greater than that elicited using either soluble antigen or conventional adjuvant approaches. The oral immunization protocol consisted of orally feeding each of the three sizes of microspheres, each containing 500 ∝g BSA, on three successive days. Interestingly, oral administration of the loaded microspheres showed that the 200 nm and 500 nm sized particles elicited fewer antibodies than an administration of antigen with either alum or Freund's adjuvant. The greatest production of serum IgG was

demonstrated using the 1000 um size particle and this group contained the higher percentage of individual responders. Analysis of serums at weeks 3 and 5 following immunization did not reveal differences in IgG2a/IgG1 isotype profiles, the latter being indicative of Th1/Th2 subset immunity and antigen-presenting differences. Ultimately, no differences were found among the various sized particles, suggesting that the method of antigen presentation was the same for all of the sizes tested. Again, the larger particles provided the higher immunoglobulin production, regardless of the mode of immunization. These results are interesting from the perspective of what has been reported [87, 88] regarding the effect of size and nanoparticle biodistribution, and what is known about the distribution of effector cells within the GALT. The present experiments used particles that were larger than those previously published; it is likely that the biodistribution affected the manner in which particulate antigen was presented for the induction of an immune response.

4.1. Targeting M cells in the GALT

Directed PLGA nanoparticles, using lectins to bind onto target sugar residues, has been shown to be a means to achieve organ targeting for the induction of a systemic immune response. In one study, PLGA nanoparticles were created by the double emulsion method and loaded with hepatitis B surface antigen (HBsAg) [95]. Lectin directed to α-L- fucose residues, *Tetragonolobus purpureas,* was bound to the nanoparticle using 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide to produce TLA lectin-PLGA-HBsAg nanoparticles that were measured to be 270 + 23nm in size. Confocal microscopy confirmed binding of the particles to the M cells of the Peyer's Patches within immunized mice. Further, lectinized particles were stabilized by the addition of hydrophilic trehalose, which improves the release of antigen. Therefore, nanoparticles stabilized with trehalose demonstrated an increased antigen release of 43.2+2.7% after 35 days, as opposed to a release of 32.4 +2.3% by the non-stabilized equivalent. In this investigation, 10 mg of encapsulated antigen per dose was used for the oral immunization of 8 week old Balb/c mice, followed with a booster 2 weeks following the primary immunization. Thus, HBsAg -loaded PLGA nanoparticles, TLA lectin-PLGA-HBsAg nanoparticles, trehalose-stabilized HBsAg -loaded PLGA nanoparticles, and trehalose-stabilized TLA lectin-PLGA-HBsAg nanoparticles were compared for the induction of antibody. Significantly, this study demonstrated the successful induction of antigen-specific IgG antibody by each of the engineered nanoparticles as determined by ELISA assay of the immune seras, when compared to the levels of antibody produced by the animals immunized with an alum based antigen. Isotyping of the antibodies demonstrated induction of IgG1 antibody, indicative of a Th2 response, at levels that were twice those attained by IgG2a which is indicative of a Th1 response. While demonstrable levels of the Th1 cytokines, IL-2 and γ-IFN, were detected in the spleens of all nanoparticle-treated animals, greater levels of γ-IFN were obtained with TLA lectin-PLGA-HBsAg, with or without stabilization by trehalose. It is not known whether the engineered particle could have induced a greater γ-IFN response as PLGA nanoparticles without antigen were not used for comparison in this study.

A directed approach has been extremely successful using chitosan alginate microparticles [94]. In this instance, chitosan nanoparticles (CNP) prepared by the ionic gelation method were

loaded with BSA test antigen, and coated with alginate. Thus, alginate was modified by using 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide to form amide linkages between the carboxylate residues on alginate and the amino group of the lectin *Ulex europaeus* agglutinin (UEA-1). The lectin *Ulex europaeus agglutinin* (UEA-1) was used to direct the microparticles towards the α-L- fucose residues found on the surface of M cells. Confocal microscopy confirmed the targeting; punctate staining was visualized using the lectin-modified microspheres. The conjugation and loading resulted in a particle shift in size: the particle size of the original CNP particle is reported as 257+ 55.17 nm, while the lectin-modified antigen carrier CNP particle size increased to 1485 + 214.3 nm. Oral immunization of 6-8 week old Balb/c mice with each of the preparations and control antigen provided striking differences in the antibody responses against BSA antigen. The highest IgG titers were obtained using alum-absorbed BSA as the immunogen's positive control, and the lowest titers were obtained using BSA loaded CNP. In contrast, antigen encapsulated in lectin-modified alginate chitosan particles (LACNP) consistently generated IgG titers that were greater than those obtained with CNP or ACNP formulations. Demonstrable levels of antigen-specific IgG2a/IgG1 were detected with all three formulations. Significantly, the highest titers of antigen-specific IgG were obtained with lectin-modified microspheres, and the results seem to indicate that there was a greater IgG2a, or Th1 response, to antigen (BSA) with that particle. The original CNP particle and the alginate chitosan particles seemed to have induced a greater Th2 response.

Together, these studies demonstrate the induction of a Th1/Th2-induced immunity using engineered particles as do others [93]. However, it is not known whether α- fucose residues are found on macrophages and dendritic cells present at other body sites, possibly resulting in multiple pathways of immune responsiveness. As discussed earlier, Unanue and coworkers demonstrated distinct differences in macrophage effector function based on the anatomical source of the macrophage [69]. Further, while TLA lectin-PLGA-HBsAg nanoparticles induced the production of sIgA in saliva and gastrointestinal fluids, it was not reported whether the engineered particles in these reports ultimately interacted with $CD11^+$ dendritic cells. IgA has been reported to be induced by regulatory T cells that have been activated by $CD11^+$ dendritic cells [50]. Finally, the directed attachment of the particles to the endocytotic M cells of the epithelial layer presents the possibility that the particles were transcytosed by the M cells towards $CD103^+$ dendritic cells, found beneath the epithelial layer. In that case, the possibility exists for the induction of tolerance [86]. Normal exposure to ingested, digested antigen results in the production of regulatory T cells that suppress an immune response in an antigen specific manner, resulting in tolerance and preventing food allergy. However, the targeted microparticles document the induction of Th1and Th2 responses.

5. Future consideration: Ingested nanoparticle and immune allergic dysfunction to foods

Proteins used in commercial food applications include casein, whey protein, collagen, egg white, and fish myofibrillar protein, and popular plant-based proteins including soybean protein and wheat gluten [7]. Compounds such as polysaccharides and proteins that are al-

ready in use within commercial food applications are attractive candidates for the production of new nanocomposite packaging and encapsulation material, as several are generally regarded as safe and are biodegradable. However, food allergy has emerged as a growing health problem throughout modern society, and current research efforts towards the identification and characterization of clinically relevant food allergens are critical to our understanding of their role in the immunopathogenic mechanisms involved in hypersensitivity reactions, and the safety of novel and proposed food-oriented nanotechnology. Thus, the characterization and identification of the proteins responsible for immune-mediated food allergies is critical.

In view of reported differences with respect to nanoparticle size and organ biodistribution, it is interesting to note that particles are often detected below the epithelium of the gastrointestinal tract. Since the Intraepithelial lymphocytes (IEL) reside below the epithelial layer of the mammalian gastrointestinal tract, an understanding of the interactions between particle and resident IEL is crucial. Following the ingestion of food, digested protein fragments, or antigens, cross the epithelium to be processed and presented on the surface of class II molecule- bearing antigen presenting cells for recognition by specific TCR-bearing T cells. Allergic sensitization in the presence of IL-4 results in the generation of Th2 cells that will assist the development of IgE^+ B cells. A repeat encounter with the antigen will result in a food allergic response. This event generates a skewed Th2 response, and will occur when luminal antigen is introduced to IgE bound onto IgE Fc receptor on the surface of mast cells. Thus, crossing the epithelial barrier to reach the mast cells is a critical step. The binding of antigen to the receptor-bound complex will result in the release of histamine, serotonin and prostaglandins in anaphylactic reactions including those generated by food.

Recent studies suggest that intestinal epithelial cells play a central regulatory role in determining the rate and pattern of uptake of ingested antigens. This is particularly critical in food allergy within the antigen-sensitized gastrointestinal tract. Studies using rats sensitized to horseradish peroxidase (HRP) showed that intestinal antigen transport is keenly affected by antigen-specific sensitization and is composed of 2 phases. The first phase consists of the rapid transepithelial transport of specific antigen from the lumen, via endocytosis, into the lamina propia. This phase is antigen specific, implying the existence of an antigen -specific receptor on the surface of the epithelial cells, and occurs within 2 minutes in sensitized rats as compared to a transit time of 20 minutes in non-sensitized, normal control animals. This is followed by a flow of the antigen in tight junctions resulting in an increase of antigen across the tissue. The second phase of antigen transport is not antigen specific, but is markedly increased by antigen challenge in sensitized rats compared with non-sensitized controls [98], indicative of the paracellular penetration through the epithelium by antigen. These studies clearly demonstrate that the kinetics of transport of antigen during IgE-mediated reactions in the gastrointestinal tract is markedly increased across the epithelium. The result of this transport is the generation of a Th2 response.

Finally, a feeding study using mice orally sensitized to the known milk allergens, casein, β-lactalbumin, and β-lactoglobulin, provided compelling evidence regarding the importance of the form of the antigen (soluble vs. particulate) for the induction of anaphylaxis [99]. The

soluble proteins, β-lactalbumin, and β-lactoglobulin, resulted in anaphylactic reactions when administered orally. Interestingly, the soluble proteins were detected in the lamina propria of the small intestine of sensitized mice indicating that these proteins were able to transcytose through the enterocytes *in vivo*. This observation was confirmed *in vitro* using Caco-2 cells. Further, the challenge with sensitizing antigen resulted in significant levels of serum IgG1, and low, but detectable levels, of serum IgE and IgG2a. Casein, normally present within micelles, demonstrated a significant difference in anaphylactic induction. Oral administration did not induce anaphylaxis. Instead, casein required a systemic administration (i.p. injection) in order to induce anaphylaxis; and it induced significantly higher serum IgE and IgG1 (Th2) allergic responses as compared to the soluble milk allergens. Further, transcytosis by casein through Caco2 monolayers was poor compared to the soluble milk allergens. When the tissue was examined by fluorescence microscopy, the casein was detectable in the Peyer's patches. Thus, these data indicated that the form of the sensitizing antigen was critical to the induction of an anaphylactic response. Next, the soluble allergens, β− lactoglobulin and soluble α-lactalbumin, were next converted into particulate aggregates by pasteurization; the process reportedly abolishes the monomeric form and supports the formation of aggregates of approximately 670 kDa. Pasteurization does not alter casein, and it exists in two predominant types as it would in its natural state: 180 kDa and 670 kDa. The conversion of soluble β− lactoglobulin and soluble α-lactalbumin into particulate aggregates by pasteurization altered the immunogenicity of the proteins such that they now required a systemic administration to induce anaphylaxis. Oral administration of either protein aggregate in sensitized mice did not induce anaphylaxis. The magnitude of the elicited serum IgG1 and IgE immunoglobulin production was much greater than that induced by their soluble forms. Further, the proteins were now detectable in association with the Peyer's Patches. Casein's induction was not altered by the process. Taken together, these results present the critical role of antigenic structure and its uptake across the epithelium as critical factors contributing to the allergic state.

The allergic state presents serious challenges to the incorporation of nanoparticles in food and food-associated products, particularly when considering the composition and ultimate biodistribution of the particles. The engineering of nanoparticle containing materials implicated, related, or identified as allergens raises concern for the initiation of alternate allergy-inducing pathways in the host. For instance, while casein is incorporated in a variety of foods and is generally regarded as safe, it is also known to elicit strong allergic responses in afflicted individuals with dairy intolerance. Disruption of the epithelial barrier is known to result in gastrointestinal illness [100]. Infection and inflammation are conditions associated with a disruption of the epithelial layer leading to the increased paracellular transport of luminal antigen. Cytokines such as IFN-γ and TNF-α directly affect barrier function of the epithelium, the latter being implicated in milk allergy [101-103]. Thus, the transit of nanoparticle through a sensitized gastrointestinal system might result in a more complicated scenario, depending upon the sensitizing antigen and the composition of the nanoparticle itself. Thus, casein nanoparticle constructs, with or without targeting lectins, might not be advisable for individuals with casein sensitivity. In this instance, the transit of the nanoparticle might be hastened across the layer, due to the pre-existing sensitivity, resulting in in-

creased transit through the layer, perhaps overwhelming the resident macrophage phagocytic activity (Figure 4), leading to exacerbation of the allergic state or the generation of alternative immunologic reactions.

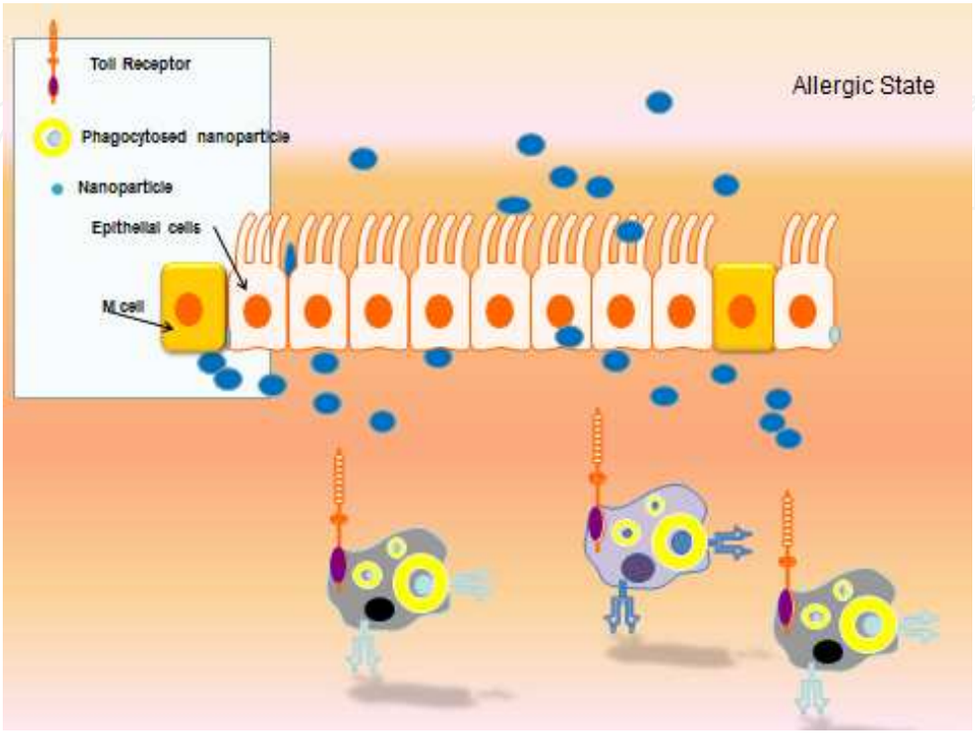

Figure 4. Schematic representation of nanoparticle transit through the epithelial layer and the allergic state. An increased rate of transit by the nanoparticles is theorized as a result of the induction or presence of an allergic state.

6. Conclusions

The choice of material used in the formulation of nanoparticles and spheres during the formulation of encapsulated nutrients or supplements intended for ingestion can be critical to the possible outcomes in mucosal immunity. A crucial consideration is whether the material will influence the induction of either tolerance or active immunity to the introduced nutrient as a result of its deposition within the gastrointestinal tract and possible interaction with resident effector cells.

The specific targeting of the nanoparticles and spheres using specific ligand interactions provides an advantage in this respect. While polymers containing natural biodegradable materials such as chitosan, PLGA, whey, casein, and others offer great advantages within this technology, they also present further challenges towards an understanding of the mechanism involved in the maintenance of gastrointestinal immune homeostasis, and preventing the induction or potentiation of immune dysfunction.

Abbreviations

GALT, gut-associated lymphoid tissue; Ig, immunoglobulin; M cell, microfold/membranous cell; CD, Cluster designation; sIgA, surface IgA; MHC, major histocompatibility complex; BSA, bovine serum albumin; PP, Peyer's patch: MLN, mesenteric lymph node

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Author details

MaryAnn Principato

Address all correspondence to: maryann.principato@fda.hhs.gov

Food and Drug Administration, CFSAN/OARSA, Laurel, MD, USA

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