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VACCINE PARTICLES WITH ANTIGENS IN LARGE NUMBERS, AND SMALL NUMBERS OF TRANSPORT PEPTIDES WHICH TRIGGER INTAKE AND PROCESSING BY MALT PATCHES

BACKGROUND

This invention is in the field of vaccines, and relates to a method for designing and manufacturing vaccine particles made of engineered microbes (such as harmless bacteriophages), having "MALT-targeting" peptides (which were identified, isolated, and sequenced, by screening billions of candidate phage particles, in a phage display library) which will cause these vaccine particles to be rapidly taken in specialized immune cells on mucosal membrane surfaces, leading to maturation and antigen-processing by dendritic cells. Without requiring any irritating or inflammatory "adjuvant" compounds (as required by injected vaccines), topical mucosal vaccines (which can be embedded in lollipops, lozenges, and similar delivery vehicles) can trigger the formation of both internal IgG antibodies (the classic Y-shaped antibodies most people already know about), as well as secreted IgA antibody dimers in saliva, nasal, lung, and digestive fluids (which very few people have ever heard of, but which play major roles in fighting off mucosal pathogens). As a result, these types of MALT-targeting mucosal vaccine particles can provide better protection than injectable vaccines can provide, while also eliminating the need for injections, needles, adjuvants, and refrigeration.

Summary of Background Section

This invention is extraordinarily complex, and the achievements and opportunities it has opened up have defied the efforts of numerous scientific teams, for decades. As a result, this Background section needs to address and summarize several different areas of science and medicine. Accordingly, this initial section is intended to offer a map, overview, and summary, of how the main parts of the Background section will be sequenced, and connected to each other. For non-experts, it is offered as a first pass through a number of important terms and topics, so that when they encounter those terms again, they will seem more familiar, and memorable. For any experts who bother to read a Background section, it will begin pointing toward certain particular aspects of immune systems, immune responses, and vaccines, which will need specific attention in order to understand the invention described herein, while also creating an assembled structure they have not encountered previously.

The first subsection below – after this "Summary of Background" section, and a "Sources for More Information" section – discusses "mucous" membranes (also spelled as mucus, and also

called mucosal). As described in more detail below, mucous membranes are those membranes, in animals, which must be kept wet, to remain comfortable and functional. They are present inside the mouth, nasal cavity, and throat; they include all tissues which are directly contacted by air, in the trachea, bronchial tubes, and lungs; they also are present in the eyes, and in the genitals and urinary tracts; and, they form the internal surfaces of the digestive system (or, at least, the esophagus, and the intestines; many people do not regard the "stomach lining" as a "mucous membrane", since it belongs in a different functional category, which must withstand the highly acidic liquids in the stomach).

For a number of reasons discussed below, mucous membranes are very tempting targets, for a huge and wide range of pathogenic microbes. With the exception of a few blood-borne diseases which are spread by insect bites, nearly all of the infective pathogens anywhere on this planet have evolved in ways that allow them to latch on to, and invade, one or more types of cells that are located in mucous membranes. All of the so-called "upper respiratory tract" infections (including cold, influenza, and corona viruses, plus pneumonia, tuberculosis, etc.); plus, nearly all intestinal and digestive pathogens (including cholera, dysentery, polio viruses, etc.); and, essentially all sexually-transmitted diseases (including syphilis, gonorrhea, chlamydia, and herpes, hepatitis, and AIDS viruses) are caused by pathogens which can latch onto, and penetrate into, at least some types of epithelial cells on the surfaces of mucous membranes. There also are hundreds of other diseases which are not thought of as "respiratory tract infections," since they mainly affect other tissues, but which are transmitted by mucosal infections. As examples, smallpox, chickenpox, and measles are all carried by tiny droplets of water that are exhaled, coughed, or spit out; those infections begin when other people inhale those microbes, which penetrate into mucous membranes, and then travel to other tissues.

Since mucosal pathogens are the most common and widespread category of pathogens on this planet, the immune systems of animals responded accordingly, by creating a "first line of defense" against such pathogens, directly on the surfaces of the mucous membranes. This "first line of defense" includes specialized surface-mounted tissue patches, called "MALT patches", which stands for "mucosal-associated lymphoid tissue" patches. Although they qualify as "lymph nodes" under the standard definition of that phrase, they usually are not called lymph nodes, because they have specialized structures and functions that do not occur in conventional internal lymph nodes.

To adequately understand those types of surface-mounted lymph nodes, one must first understand the normal types of lymph nodes that are located inside the body; and, the best way to develop a working knowledge of how lymph nodes function, inside an animal body, is by pointing out a crucial similarity between how lymph nodes function in animals, and how armies design and build forts, when they are trying to establish control over a hostile frontier, or enemy territory. Such forts need to be able to respond, immediately, to any type of attack, without having to wait until additional supplies, reinforcements, soldiers, or instructions arrive from some distant government location. Therefore, they need to be provided, in advance of any attack, with all of the manpower, weapons, ammunition, building and repair tools, and other supplies

they will need, to fight off not just one attack, but a series of predictable attacks. And, the soldiers in those forts can perform much more efficiently if they have been properly trained, so that different soldiers with different specialties (such as infantry, snipers, and artillery) can all work together, in coordination and cooperation.

In similar ways, the lymph nodes in an immune system are where all of the major types of immune cells (including B cells, T cells, macrophages, and dendritic cells, as described below) are gathered together, so that they can work together, quickly and efficiently, to do whatever needs to be done, in active cooperation with each other, to defend against any type of invader that is trying to invade the body and set up its own pockets of strength.

Once that basic concept is understood, the next step is to analyze how normal (internal) lymph nodes had to change and adapt, to render those which are mounted on the surfaces of mucous membranes, better adapted (and better able) to protect those mucous membranes.

Surface-mounted "mucosal-associated lymphoid tissue" (MALT) patches are subdivided into different groups, depending on where (inside a body) they are located. One major (and easily-accessible) group is called NALT patches, where N stands for nasal (or, more accurately and broadly, "naso-pharyngeal", which also includes the "pharynx", which is where the passageways of the nasal cavity, mouth, and throat come together, in the back region of the mouth). The two other main subtypes of MALT patches are called GALT patches (G stands for gut; GALT patches include all MALT patches in the intestinal tract, which also are called "Peyer's patches"); and, BALT patches (B stands for bronchial, and the BALT group includes all MALT patches in the trachea, bronchial tubes, and lungs).

The main adaptation that emerged, as MALT patches evolved over time, centered on a set of surface-exposed sampling or "gateway" cells, called "M cells", which became crucial components of those surface-mounted lymph nodes, with not just one but an entire set of remarkable functions. If a particle contains a protein having a "pathogen-associated pattern" on its surface, an M cell can actively grab that particle, and pull it inside, using a process called "phagocytosis", which keeps the pathogen sealed and isolated inside a protective membrane bubble. It will then pass that particle (and the membrane bubble which encloses it) rapidly through the M cell, using a specialized process which only M cells perform, called trans-cytosis (which translates into "through the cell", or "across the cell"). It will then eject that particle out of the M cell, in a non-enclosed "naked" form once again, through a "back door" in the M cell, into a "docking site" located on the "backside" (or basal, underside, posterior, hidden side, etc.) of the M cell.

Those "docking sites", on the back sides of M cells, are one of the key places where immature "dendritic cells" go to, before they go through an activation and maturation process which will cause a specific immune cell to commit itself to helping fight and destroy a single particular type of pathogen. When an immature dendritic cell reaches a docking site, on the back (basal) side of an M cell, it will simply sit, stay, and dwell there, until that M cell pulls in a particle which appears to be a pathogen, and then passes it rapidly through the M cell, and then ejects it, directly into the "waiting arms" of that immune cell. Dendritic cells use large numbers

of surface receptors to “analyze” particles which may be pathogens, and decide which ones are important enough to trigger a complex, multi-step response which will require the dendritic cell to leave that spot, travel to the “germinal center” of a lymph node, and offer up “chunks” of the surface protein it has semi-digested, from the particle it processed while it was traveling, to a combination of B and T cells. Those B and T cells will then take over, and create antibodies which will bind to those “chunks” of proteins from the invading particle (alternately, they sometimes respond by activating “killer T cells”, but that alternative process is not of interest herein).

By contrast, if a particle which has been delivered (by an M cell) to a dendritic cell which is waiting for a “pathogen delivery” in the “docking site” of an M cell, does *not* merit that type of “antigen processing and presentation” response, the dendritic cell will simply ingest it, and break it down into its building blocks, without committing to the “antigen presentation” process, and without traveling to a lymph node.

And, as a crucial additional fact which will be described in detail below, not-yet-activated dendritic cells are able to simply wait, patiently, in a docking site behind an M cell, for as long as it takes to receive an apparently important and dangerous-looking pathogen which merits a full antibody-triggering response, because of a key factor, which undergoes a dramatic change, as soon as a dendritic cell is “activated” in a way which triggers the “maturation” process. Immature dendritic cells carry multiple copies of a special type of surface receptor which can response to signals from external “chemo-attractant” molecules, in a manner which will guide the travel (or “migration”) of an activated (or “maturing”) dendritic cell, to the “germinal center” of a lymph node, which is where B and T cells wait for antigen deliveries. However, those “motion-triggering” surface receptors remain hidden inside a dendritic cell, for as long as that dendritic cell remains in its immature state. Then, if and when a dendritic cell determines that some candidate particle – which has been handed off to the dendritic cell, by an M cell – appears to be a dangerous pathogen, the dendritic cell will respond by “translocating” those receptors to its exposed outer surfaces. That will enable those receptors to be directly contacted, and activated by, certain types of chemo-attractant molecules from a lymph node. When that happens, the dendritic cell will then begin moving, and will leave the docking site beneath an M cell, and will go off in search of a germinal center, in a lymph node, by always heading in the direction where the highest concentration of those chemo-attractant molecules are coming from.

That factor is being mentioned here, in this Background section, because it is a long-known fact, about dendritic cells. By contrast, the insight and the methods and reagents which sit at the heart of this invention, and which enabled the Inventor herein to conceive, create, develop, test, and use a screening test which can powerfully and efficiently use and exploit that change in dendritic cell status, to identify, isolate, and determine the peptide sequences of a small number of potent “MALT-targeting” transport peptides, out of a billion candidate phage particles – is described farther down, in the Summary of the Invention and Detailed Description sections, since it is part of this invention.

After the above-summarized information (focusing on mucosal membranes, lymph

nodes, MALT patches, M cells, and dendritic cells) has been covered in this Background section, the focus will shift over to the differences between two very different types of antibodies, which have entirely different structures, because they must perform entirely different functions.

As a brief preview, the classic Y-shaped antibodies, which nearly everyone learns about (or has at least heard about, and seen pictures of), can only function *inside* the body of an animal. Anyone reading this patent application will almost certainly already know that the two "arms" of a Y-shaped antibody will bind, selectively, to pathogens. However, very few people know what happens, after that binding reaction occurs. The "stem" of a Y-shaped antibody (i.e., the "trunk" portion, which holds together and supports the two branching arm portions) used to be called the "fragment constant" (Fc) portion, because all IgG antibodies, in any specific animal, have the same (constant) amino acid sequences. However, scientists learned, years after that "constant" name was adopted, that the stem portion of a Y-shaped antibody could more accurately be called a "shape-shifting fragment". Instead of being "constant", it undergoes a crucial and dramatic change in shape, when that antibody binds to a pathogen.

Before an antibody binds to a (presumably pathogenic) particle, the Fc "stem" portion functions as a signal, which effectively tells any and all cells, "Leave me alone. I'm an unattached antibody, and I have a special job to do, so just ignore me". Stable unattached antibodies can last for months, and possibly even years, inside an animal body.

However, after a pathogen-binding reaction occurs, the so-called "constant fragment" (i.e., the stem portion) of a Y-shaped antibody changes shape. It changes from a "leave me alone" shape, into a very different shape, which effectively becomes a combination of a loud siren, and bright flashing lights, to attract the attention of any mobile immune cell that happens to be in the vicinity. The first mobile cell which can get to that "immune complex" (i.e., the combination of a foreign particle, and any antibodies which are attached to that particle) will engulf and begin processing both the particle, and any antibodies attached to that particle. At that point, an antibody's work, and function, are finished and done. Any such antibody will be pried off of the particle, and then digested, by the immune cell, to get the antibody out of the way, so that the immune cell can then focus on, and begin processing, whatever the antibody had attached to.

As a brief aside, once the scientists realized that what they had called the "Fragment constant" (Fc) portion of an antibody, was actually a "shape-shifting" segment, they renamed it the "Fragment crystallizable" segment, so that they could continue to use the same acronym, i.e., "Fc". It would be interesting to find out whether any scientists have ever tried to actually "crystallize" a batch of Fc fragments . . . which would be a challenge, because they have globular shapes, and will not fit together like a normal crystal.

The classic and familiar Y-shaped antibodies – which can function properly, only inside a body – are called "IgG" antibodies, an acronym for "Immuno-globulin, type Gamma". As an aside, the directly-analogous Y-shaped internal antibodies in birds, reptiles, and amphibians (all of which hatch from eggs) are called IgY antibodies, rather than IgG antibodies, where the "Y" reference arises from yolks; and, any references or discussion herein which refers to "IgG"

antibodies is also intended to apply to IgY antibodies, in birds, reptiles, or amphibians). IgG antibodies travel through soft tissues, in both circulating blood (i.e., inside blood vessels), as well as through "lymph."

Another digression is needed to define the term "lymph", since different experts use it in two different (and conflicting) ways. As used herein, "lymph" includes any and all of the clear and watery liquid which surrounds and bathes the cells. It enables nutrients to reach the cells, and it also helps metabolites diffuse away from the cells, in soft tissues. Instead of being merely thin layers on the surfaces of cells, that watery extra-cellular fluid takes up about 1/6 of the volume, in most types of soft tissues, and the gaps between many neighboring cells are large enough to allow "mobile immune cells" (which can travel in a manner comparable to an amoeba, as discussed below) to squeeze and travel through those gaps.

However, it should be noted that *some* experts limit and restrict the term "lymph," to include only those watery extra-cellular fluids which have already reached and entered a "lymphatic drainage channel."

Most people who are likely to read this application do not need to worry about that subtlety, since it does not affect this invention; so, "lymph" is used herein to include any watery liquid that mobile immune cells (also called lymphatic or lymphoid cells) travel through, regardless of whether some specific volume or molecule has or has not reached a lymphatic drainage channel.

As another brief aside, IgG antibodies do not travel through the brain or spinal cord, where the watery extra-cellular fluid (with no antibodies or immune cells) is called "cerebrospinal fluid" (CSF). Rather than using an "immune system", CNS tissue generally uses a class of non-neuronal cells called "glial cells", for defense and maintenance. The term "glial" arises from the same root word as "glue"; once they had created microscopes, but before they knew how the cells they were seeing actually worked, anatomists thought glial cells merely acted as a "glue" which held the neurons in place. There are about 5 or 6 major types of glial cells (depending on how someone defines the groupings), and that term includes any and all non-neuronal cells, in brain or spinal cord tissue. They handle any and all "housekeeping and maintenance" functions, to help the neurons focus on handling nerve signals; and, they usually try to either ingest and dissolve, or surround and "wall off", any intruding microbes or particles, without using antibodies (those processes are usually called "reactive gliosis").

Returning to the main topic, IgG antibodies do not carry any toxins, tools, or other "defensive weaponry"; instead, all they can do is bind to foreign particles, and then change the shapes of their "butt ends", which will protrude outwardly (in much the same way that, if an ant sinks its jaws (or mandibles) into something and will not let go, the hind end of that ant will "stick out"). That action will recruit an immune cell to come over, take charge, figure out what needs to be done, and then begin doing it.

Actually, and more precisely, there is an entire set of important proteins, called "complement proteins", which can respond much more rapidly to a "shape-shifted" Fc portion of an antibody which has become bound to something. Complement proteins are much, much more

plentiful than immune cells; so, they usually show up first, when an antibody binds to something and goes through its shape-shifting process. Complement proteins will bind to the shape-shifted Fc portion of an IgG antibody which has become part of an "immune complex", and when they do, they do their own shape-shifting, which converts them from an inactive precursor form, to a highly-active signaling form. The actions of complement proteins "amplify" the actions of shape-shifted IgG antibodies, and mobile immune cells usually respond to complement proteins that have attached to antibodies, rather than binding directly to the antibodies themselves.

That type of "recruitment, of immune cells" is what happens *inside a body*, where immune cells are available to do that kind of work.

By contrast, antibodies which have been secreted into saliva, or into the other types of mucosal fluids that coat the surfaces of mucosal membranes, cannot work in that way, for the simple reason that there are not nearly enough immune cells available, in secreted (i.e., outside of any cells or tissues) mucosal fluids, to be able to offer any substantial help, to any antibodies that have attached to something and changed the shapes of their stems. Although there are minor levels of leakage of small numbers of immune cells into secreted mucosal fluids, there simply aren't any significant numbers of the types of immune cells which can respond to some type of "come over here and help me" signal, from an antibody which has changed shape after attaching to a foreign particle. Therefore, the antibodies that are secreted into saliva, and mucosal fluids, had to evolve with very, very different shapes, and functions.

Those types of antibodies are called IgA ("Immuno-globulin, type Alpha") antibodies. That abbreviation usually is preceded by an S (either lower or upper case S, and either hyphenated, or not), which represents the word "secreted", to create the acronym "sIgA" (or SIgA, or S-IgA, etc) which refers to IgA antibodies that have been secreted by a mucosal membrane, and are outside of any cells or tissues, in saliva or mucosal fluids.

Two crucial and central facts need to be understood, by anyone who wants a basic understanding of the "mucosal" part of an immune system. Those two facts are:

Crucial Fact #1 re: Mucosal Antibodies: Every sIgA antibody is actually a "dimer" antibody, formed when a cord-like protein is wrapped around the "stem" portions of two T-shaped antibodies, by a mechanism described below. That creates an elongated cluster, having four different "sticky arms" (with two such arms, at each of the two ends of each antibody "dimer"), provided by the Fv "variable fraction" portions of the two antibodies which were strapped together, to create a single IgA dimer. That allows sIgA antibody dimers to be "extra-sticky", and their main task is to grab hold of any particles that might be pathogens, and drag them down into the throat, and then into the stomach acid, which can kill most types of microbes. Those extra-sticky antibody clusters also help prevent microbes from using the tactics and proteins they developed, over the eons, to latch onto and then penetrate into, and infect, the cells on the surfaces of the membranes.

To help readers understand and remember that crucial difference between both the shapes and the functions of IgG antibodies, versus IgA antibody dimers, two phrases have been adopted and used herein: Y-shaped internal IgG antibodies use a "*tag and flag*" process, to recruit

immune cells to come help out, while secreted IgA dimers use a "*grab and drag*" process, which does not require any immune cells to help.

Crucial Fact #2 re: Mucosal Antibodies: The second crucial factor which any reader should be at least aware of, when trying to grasp the potential importance of a new class of mucosal vaccines, is this: *there are more than twice as many of the IgA antibodies dimers,* in just the few pounds of mucosal fluids in any normal adult, *than the total number of Y-shaped IgG antibodies, in the entire remaining body, and bulk, and weight, of that adult human*. That ratio is a remarkable number, and it deserves to be re-stated, using different words, to emphasize it adequately. *In just the thin layers of saliva and mucous* which coat the surfaces of the mucous membranes (*those liquids likely weigh less than about five pounds, total,* in nearly any healthy adult human), there are *more than twice as many secreted IgA dimer antibodies, as there are IgG antibodies in the entire remaining mass and weight of that adult human.*

That "*more than twice as many*" ratio is remarkable; it merits serious attention, and it must be considered alongside the equally important fact that pathogens which can attack and infect mucosal surfaces are the largest and most important functional group of pathogens, anywhere in the world.

Those two facts, taken together, are clear and unmistakable indicators that the mucosal portion of any mammalian immune system is a huge, crucial, and critical part of a complete and overall immune system. Otherwise, why would animal bodies devote so much time, energy, and resources, to creating so many more secreted IgA antibody dimers, in just those thin liquid layers, than all of the IgG antibodies that are present throughout the entire remaining mass and weight of the body?

Consider, also, a basic and crucial fact about viruses that can infect cells on the uppermost surfaces of the nasal cavity. The cells that cover the surface of the "epidermis" (i.e., "dry skin", as opposed to mucous membranes) are not complete cells. Instead of being formed by cell division, they are formed by a "budding" process, in which special precursor cells, about 6-8 layers deep in most areas, rapidly enlarge to nearly twice their normal size, and then "pinch off" something which is basically an empty bag – with a normal outer membrane, but with almost nothing but slightly salty water inside that membrane. That makes epidermal cells ideal as "decoys" or "bait" for pathogens, which use their tricks and tools to break into those cells, only to discover that they have broken into an empty bag, with none of the things they were looking for – none of the things they need, to reproduce, and none of the things they need, to escape.

By contrast, unlike the empty-bag pseudo-cells that cover the epidermis (dry skin), the cells on the surfaces of the membranes inside the mouth and nasal cavity (and on all other mucous membranes) are complete and fully functioning cells, with all of the biochemical machinery and building blocks that pathogens need, and use, to make copies of themselves. Those cells are called "epithelial" cells, and for multiple reasons, they are very tempting targets, for any type of pathogen which has evolved with some type of mechanism (and there are dozens if not hundreds of such mechanisms) for grabbing hold of an epithelial cell, and then invading that cell.

If influenza or corona viruses (or other viral pathogens) infect the very top layer of cells, on the surfaces of a membrane inside a mouth, throat, or nasal cavity, they usually have no contact, whatsoever, with any internal IgG antibodies that are traveling through and policing the lower levels of tissue, beneath that outermost layer of exposed and vulnerable cells. To a very large extent, it simply does not matter whether a person who is trying to fight off that type of surface-cell infection has an abundant supply of internal IgG antibodies, deeper down inside the soft tissues, in his or her blood and lymph, after being vaccinated against (or previously infected by) similar or even identical influenza or corona viruses. IgG antibodies must remain inside the blood and lymph, in soft tissues, and can have only limited contact with the undersides of the uppermost surface cells. Since viruses can replicate so rapidly, by the time an infected mucosal surface cell can manage to move distress-signaling molecules onto a lower surface, to try to gain the attention of any immune cells in the layers beneath it, and then wait for an immune cell to respond by performing all of the steps that will enable that immune cell to engulf and disable the infected cell on the mucosal surface, the viruses inside that cell are likely to have already spewed out hundreds of copies of themselves (usually by "lysing" (bursting open) the infected cell), into the mucosal fluids above the membrane, where that new batch of virus particles will be beyond the reach of any IgG antibodies, or any immune cells.

That combination of factors explains why, even among people who have been fully vaccinated (and boosted) against corona viruses, and/or who previously were infected and then had plenty of time to fully recover, very high "loads" of fully infective, dangerous, pathogenic influenza, corona, or other viruses can be (and usually are) present, in their mouths and nasal cavities, floating freely in (and spreading dangerously and contagiously from) their saliva and nasal fluids.

Scientists have known those things for decades. They also have known, for decades, that if good and reliable vaccines could be created which would trigger and drive the production and secretion of large numbers of mucosal IgA antibodies into mucosal fluids, then a combination of both: (i) substantial numbers of internal IgG antibodies, inside a body; and, (ii) substantial numbers secreted IgA antibodies, in saliva, nasal fluids, and other mucosal fluids, would be more effective, and more protective, against mucosal pathogens, than either an internal antibody response, or a secreted antibody response, could accomplish, by itself, without the other. In the same way that people can grab, handle, and control large and heavy things much more effectively, if they can use *both* hands (instead of being limited to using just one hand, or the other), a balanced, dual-component, "two-handed" vaccine approach (which could create both: (i) IgG antibodies, inside the body, and (ii) sIgA antibody dimers, in the mucosal fluids) could be extraordinarily valuable, for medical and public health reasons, for commercial and financial reasons, and to reduce the costs of treating and caring for people who have become infected by mucosal pathogens.

Accordingly, numerous research teams, staffed by top-level immunologists and vaccine experts, have been trying for decades to create good mucosal vaccines. However, as described in more detail below, the few limited and inadequate "wins" that have been achieved, in those

efforts, are spotty, sporadic, inconsistent, and severely inadequate to meet numerous pressing needs that are still unmet, in that field of research.

Indeed, one of the great unmet goals and objectives of modern medicine, which has reached an almost "Holy Grail" status, would be to create or discover a "mucosal vaccine platform" which can be adapted, tweaked and tuned, and then used, effectively, to fight off and protect against any type of pathogen or disease which attacks mucosal membranes, by merely inserting one or more selected pathogen-derived antigen sequences, into a proper location, in an effective and potent "targeted transport" vehicle which can trigger secreted IgA production; and, as described below, that is exactly what this invention is believed to be capable of creating, and offering.

Moving on, in this summary of what is covered in the Background section below: several subsections, near the end of this Background information, describe a class of viruses that initially were called "bacteriophages", and are now usually called simply "phages".

There are two reasons for addressing "phage" particles, in this Background section. The first reason is this: bacteriophages offer a class of vaccine "vehicles" that are highly promising, for use as described herein, for not just one or two, but multiple reasons. Those advantages are described, not in this Background section, but in the "Detailed Description" section, farther down, since they are part of this invention, rather than Background information.

The second reason why phages are described in this Background section is because the research which led to this invention involved complex preparations called "phage display libraries", which were developed by others, beginning in the 1980's. Like a large library which contains more than a million books, a "phage display library" can contain literally billions of potential candidate particles, with each different phage particle containing multiple copies of a single relatively small foreign insert (usually about 12 amino acids or less) in multiple copies of one of its surface proteins. Huge numbers of candidate phage particles can be tested in "screening tests" that are designed to identify (and, in most cases, to isolate, "rescue", and make additional copies of) those particular phages which happened to perform some particular process of interest, in some particular type of cell, tissue, or animal.

The Inventor herein – a former university professor whose specialty was tracking and analyzing where different types of molecules go, after they enter an animal body – created not just one but two different types of new (and apparently never-before-tried) screening tests, which were carefully designed and carried out to identify and isolate no more than a few dozen "winners" from among roughly a billion candidates. Furthermore, after he encountered repeated frustrations and disappointments while trying to use the smallest cell-harvesting utensils he could obtain, for a highly specialized cell-harvesting operation, he ended up developing a new type of cell-harvesting tool, which is believed to be a new and patentable invention (and, potentially, a useful research tool) in its own right.

The last subsections, in this Background section, are effectively the "caboose" or "cleanup" sections. They discuss, for example: (i) "adjuvants", which are specialized compounds that are added to vaccine particles, to make the resulting formulations more effective; (ii)

"epitopes", which are the exposed portions of surface proteins, on pathogens, that make the best candidates, when candidate antigen sequences for a vaccine are being analyzed; and, (iii) the signaling molecules (called "cytokines" rather than "hormones") which immune cells use to communicate with each other, and to receive distress signals from other types of cells..

That completes an overview and summary of "the high points" that will be found in the remainder of this Background section. The next section, immediately below, describes sources for more information about the topics described below, for the types of not-yet-experts-in-immunology who might need to read this patent application (or a patent which arises from this application). Rather than being added to the end of this Background section, it is provided as the very next section, in the hope that at least some readers who encounter a new term or topic might actually go to one of those resource materials, and find out more about whatever caught their attention. The remaining sections, after that, will cover the same topics that were summarized above, but in more detail, and with citations to relevant items of background and/or prior art.

SOURCES OF ADDITIONAL INFORMATION

Because immune systems are so complex, it is impossible to describe them adequately, in words, without using illustrations, diagrams, and videos which show how various cells and molecules go through sequences of steps as they interact with each other.

However, those types of visual support are not suited for patent applications. Therefore, this section lists several sources for more information, not merely as lists, but with comments that will try to help non-experts figure out where to go next, depending on their current level of expertise, if they want to know more about any particular topic.

As two starting points, anyone who works actively in a field that relates directly to the mucosal immune system should be aware The Society for Mucosal Immunology (SMI), a professional group (formed in 1985) with a website at www.socmucimm.org. They have issued a textbook, *Principles of Mucosal Immunology* (P.D. Smith, editors, 2nd edition, 2020, CRC Press), and they also have a journal, *Mucosal Immunology* (the website of that journal is www.mucosalimmunology.org).

In addition, anyone who works in this field should be aware of the major contributions by Per Brandtzæg (1936-2016; also spelled Brandtzaeg), a Norwegian who began as a dentist, and became a Professor of Medicine at the University of Oslo. He helped organize the field, as it emerged as a separate and distinct field of immunology, and he played a major role in establishing an agreed-upon set of terms for various cell types, tissue types, and processes. An article he coauthored (with R. Pabst, in 2004) proposed a set of terms which was unanimously adopted by the SMI in 2007. That set of terms was later polished a bit more, by the Nomenclature Committee of the Society, and the formally-adopted version was published as Brandtzaeg et al 2008, entitled "Terminology: nomenclature of mucosa-associated lymphoid tissue." It is available at no cost from www.mucosalimmunology.org/article/S1933-0219(22)01651-8/fulltext. His other most important article is a review article which surveys the field, published the following year (2009), entitled, "Mucosal Immunity: Induction,

Dissemination, and Effector Functions" (available at no charge from onlinelibrary.wiley.com).

Backing up a few steps (or, more than just a few), anyone who has not previously worked with mucosal immunology needs and deserves some entry-level suggestions, on how to get started, if they want to learn more. It merits mention that entries posted at Wikipedia.com, concerning the immune system, are of generally good (indeed, surprisingly good) quality, and offer numerous helpful illustrations and introductions. Unlike postings that focus on politics, celebrities, entertainment, and other "ephemera", one can safely assume that any Wikipedia entries on cell types or immune systems were written by experts who, in good faith, want to share their knowledge with others. While experts might find grounds to quibble over some sentence or phrase in some entry, the Wikipedia entries on immune systems (and the cells, components, and processes involved) have reached a level of being useful and respectable illustrated summaries, for any non-expert who wants to reach a better understanding of the immune system; and, this becomes even more true, for readers who are told of specific words or phrases that can help them go beyond the obvious "entry level" pages, and into deeper analyses of specific cell types and other components of the immune system.

In addition, there are numerous videos describing the immune system on YouTube, and it is not difficult to determine fairly quickly whether any particular video will be on a level which any viewer is looking for, by both: (i) checking to see who made any particular video; and, (ii) viewing the first half-minute or so.

As a first citation to a set of basic-level videos, followed by a second citation to a set of much more sophisticated videos:

1. The "Crash Course Biology" series has 40 episodes listed at www.youtube.com/playlist?list=PL3EED4C1D684D3ADF, and additional episodes available via www.youtube.com/watch?v=GIJK3dwCWCw. Episodes 32 and 45-47 focus on the immune system. These episodes create the impression of a college or medical student trying to help high school seniors decide whether they really want to go into "pre-med" as a major in college.

2. A series by Armando Hasudungan, who was a medical student in Australia when he made these videos (these videos also can be found at armandoh.org/subjects/immunology/). This series is at a higher and more complex level, suited for those who have already graduated from college; indeed, they were created specifically to help medical students. In these lectures, Hasudungan (unseen) describes what is being shown, while he creates hand-drawn pictures on paper, using felt-tip pens in several colors, and using speeded-up video to keep pace with his narrative. The entire series includes 35 segments, most of which are about 10 minutes long. At the start of each video, he repeats his standard introductory comments in an all-too-rapid voice, with an "English as a second language" accent that is likely to be off-putting to some; however, after the introductory comments, he slows down and is not difficult to follow, for viewers willing to put in a bit of effort. There is no reason for anyone other than medical students or full-time biomedical researchers to plow through the entire series; however, any episode labeled as "Overview" (or, at least the first five minutes of any such episode) might be worth the time and effort for anyone who seriously wants to improve his/her understanding of the immune system.

The videos can be expanded to full-screen size, and they can be paused, when a drawing is finished, so that a viewer can "grab" a full-color copy of it by: (1) using the "Print Screen" command to "grab" the image in "Clipboard"; (2) pasting that image into a graphic program, such as Microsoft Paint; and, (3) saving that image under any desired filename, using a "minimal bulk" format such as "filename.jpg" (named after a committee called the "Joint Expert Picture Group" created that method of compressing picture files).

Various other materials also are available via the Internet. For example, the materials for numerous college or medical school courses on immunology are available at no charge via a number of websites, such as uhaweb.hartford.edu/BUGL/immune.htm, as just one example. Glossaries also are available, such as at users.path.ox.ac.uk/~scobbold/tig/gloss.html.

Anyone who prefers printed information can find "single chapter" summaries which provide good overviews of the immune system, in medical textbooks which do not specialize in the immune system (such as Alberts et al, *Molecular Biology of the Cell*, or Guyton and Hall, *Textbook of Medical Physiology*). More detailed analyses are available in textbooks which are entirely devoted to immunology, such as any recent edition of *Kuby Immunology* (Kindt et al, W.H. Freeman), *Immunology* (Goldsby et al, W.H. Freeman), *Immunobiology* (Janeway, Garland Science), or *Fundamental Immunology* (Paul, Lipincott). Outdated editions of those books are available at relatively low cost, through sources such as Amazon, eBay, and internet booksellers.

In addition, numerous review articles (which can be located through the U.S. National Library of Medicine database, via a system called PubMed, at pubmed.ncbi.nlm.nih.gov) provide more information on specific aspects of vaccines or immunology. While most such web pages contain only the title, authors, and abstract, with a link to find and purchase the entire article if one desires, quite a few articles can be downloaded in their entirety, for free, via a system called PMC (for PubMedCentral). Importantly, the ones that are available as free complete copies tend to include review articles, and articles written for audiences at the level of medical students, or for doctors who already have some particular specialty, and who are trying to learn more about some other specialty.

Non-experts also should note that PubMed will allow any user to limit any search, using the term "review", which will cause that search to list only review articles. By using that limiter, searchers can much more rapidly find articles by experts, which review and summarize numerous other published articles and developments, in any particular specialty. As examples, Thomas et al 2005 and Galen et al 2014 are review articles which cover genetically-engineered vaccines, and Gaubin et al 2003, Clark et al 2004, Wang et al 2004, Miedzybrodzki et al 2005, Prisco et al 2012, Bazan et al 2012, Hodyra et al 2015, and Aghebati-Maleki et al 2016 are reviews which focus on bacteriophages as vaccine carriers or delivery vehicles.

The Latin phrase 'et al' (which translates into 'and other authors, as well') was used above, as a gesture of respect, and acknowledgement. However, in all citations below, throughout this application, it has been deleted, to shorten and condense the references, and anyone reading this should realize that about 99% of all scientific and medical articles in respected journals are co-written by teams of authors; and, for those who might not know it, the

first author is the one who contributed the most heavily (unless some sneaky person found a way to beat or cheat the system), while the last-named author also is a place of pride, because it usually names the senior/top manager in charge of that lab, research group, or project. Complete citations (with journal names, volume and page numbers, etc.) after the examples and before the claims, near the end of this application. If any full-length citation has been inadvertently omitted, it very likely can be found by searching for the author's name, and the year of publication, at the PubMed website (cited above).

Accordingly, any background information herein on the immune system (or on any cell type, component, or process which is part of the immune system) is intended only as an introduction and overview, and more detailed information is available from other sources.

Furthermore, any sentence which appears to read as an absolute statement, along the lines of "This process ALWAYS happens", should be regarded as having escaped proper notice, during drafting and editing. Almost nothing, in biology, ALWAYS happens in a single specific way, every time, and in every species; there will (pretty much always) be exceptions, qualifiers, and leakage, as well as gray areas, boundary zones, etc., that do not fall cleanly and clearly into some specific category, or description. So, apologies are offered in advance for any too-stiff statements or summaries, and the author of this application reserves the right (hopefully, in each and all of patent law, scientific company, and common sense) to clarify any infelicitous overstatements, or inaccurate generalizations.

MUCOSAL TISSUES, AND MUCOSAL PATHOGENS

As mentioned above, this invention describes vaccine particles which can be administered intranasally (or in other "topical" ways, by contacting them with exposed surfaces, rather than by injection or other means that would insert them below an exposed surface) to "mucosal membranes." Therefore, this section will focus on what mucosal membranes are, how they function, and why they are the targets for so many different types of pathogens.

As a simple definition, terms such as "mucosal" (or mucous, mucus, etc.) refer to membranes (inside the mouth, nasal cavity, lungs, intestines, genitals, etc.) which must be kept wet and lubricated, to remain comfortable and healthy. Although most people are startled (and many don't believe it), when they hear this number, the total area of the mucous membranes, in an adult human, is estimated to be *about 200 times larger than the total area of dry skin*, which covers the body. While that ratio might seem exaggerated, or even preposterous, two factors help explain it:

(i) it includes all of the microscopically small sacs inside both lungs, when fully inflated. That surface area, by itself, is (and must be) quite large, to provide enough gas transfer, across those membranes, to supply all of the muscles, organs, brain, and other tissues of the body with enough oxygen to keep them fully functioning, even under periods of strenuous exertion; and,

(ii) most mucosal surfaces have very large numbers of folds, ripples, invaginations, and other irregularities. Some are visible, but many more are microscopic in size. Those folds, ripples, etc., provide mucous membranes with numerous advantages, including remarkable

flexibility and stretchability, as well as greatly expanded surface areas, which allow very large numbers of surface-mounted cells (and even specific types of cell surfaces) to perform specialized functions.

In addition, there is an absolutely crucial difference between: (i) the types of cells which cover mucous membranes, versus (ii) the types of cells which cover "dry skin".

As mentioned above, in the "Summary of the Background" section, cells which cover "dry skin" – epidermal cells – are not even complete cells. They have no nucleus, no DNA, no RNA, and no ribosomes (the cellular components that create proteins). Instead of being complete and actual cells, they are formed by a "budding" process, which involves specialized precursor cells, located in layers which are about 6 to 8 cell layers below the dry skin surfaces, in most locations around the body. Each precursor cell endlessly repeats a cycle, in which it enlarges to almost twice its initial size, and then "pinches off" a balloon-like appendage, in a way that gives the newly created "cell" its own membrane, containing a droplet of cytoplasm and not much else.

However, despite that status as largely empty cells, epidermal cells nevertheless contain large number of receptor proteins, on their surfaces. That helps them function as "decoy" cells, which pathogens will latch onto, and invade, only to learn – too late – that they have used up all of their resources to break into a house which is empty, and contains nothing of value, and from which they usually cannot escape.

As a result, the epidermis forms a protective layer of cells which are, quite literally, already dead, as soon as they are formed. They begin losing their water as soon as they are "pinched off" from the precursor cell, so that by the time an epidermal cell reaches an outer skin surface, it has become flat and "desiccated", comparable to a forcibly-flattened raisin which previously was a grape (the term "squamous", which rhymes with famous, also is used to describe epidermal cells which have reached that status). Roughly 50,000 dried skin cells flake off an adult's skin every hour, even during sleep, leading to an entire ecosystem of mites which are too small to see, but which (graciously) remove and process that dead organic matter for us, before it begins to smell bad.

During the few days that an epidermal cell remains on the skin surface, it helps absorb (with active involvement of the receptor proteins mounted on its surface, as mentioned above) invading viruses and bacteria, in what becomes – for microbes unlucky enough to enter and get stuck in an already-dead epidermal cell – an empty shell which doesn't have the nutrients or supplies which most microbes need, in order for them to reproduce.

By contrast, the cells which cover *mucosal* membranes were given a completely different name ("epithelial" cells), since they come from different origins, and are very different from epidermal (dry skin) cells. As a starting point, epithelial cells are complete cells, formed by full and complete reproduction of their precursor cells. As such, they contain nuclei, DNA, RNA, ribosomes, and all the other components that microbes try to hijack and turn to their own purposes.

Furthermore, rather than offering merely a brief-in-time opportunity (as occurs when a puncture wound or a cut is caused by a thorn, a sharp pebble, or the teeth of a predator or

parasite), which will heal and close up within a few days – and which therefore will offer only a brief opportunity, which will depend heavily on timing and luck – the mucosal membranes of any animal will always be present, and "reachable", throughout the entire lifetime of that animal.

Therefore, nearly all types of microbes which pose serious threats of creating diseases, among animals, evolved over time in ways which enable them to infect mucosal membranes (or, more precisely, with an ability to infect at least some types of cells, on at least some types of mucosal membranes). With the exception of blood-borne pathogens (spread mainly by insect bites), nearly all types of pathogens evolved in ways that make them excellent competitors, in their race (against each other) to efficiently attack and invade mucous membranes. All upper respiratory infections, lung infections, intestinal and digestive infections, and sexually transmitted diseases are caused by pathogens that can directly attack mucosal membranes; furthermore, numerous other diseases (including smallpox, chickenpox, and measles, as examples) also are transmitted via mucous membranes, even though their symptoms, after an infection has become established, do not involve mucosal membranes. Therefore, mucosal infections have sickened and killed more people, and other animals, throughout history, than any other class of microbial infections.

That fact, and additional facts mentioned below, offer potent and powerful statements, by both physiology and evolution, that the mucosal components of immune systems are hugely and critically important, and at least *SHOULD* merit serious and sustained attention, when it comes to vaccines. However, as described in more detail below, the history, efficacy, and availability of mucosal vaccines are sadly limited, and severely lacking.

LYMPH NODES and TYPES OF IMMUNE CELLS

As mentioned in the summary section, at the start of this Background section, it is easier to understand how the surface-mounted lymph nodes on mucosal tissues work, if one first understands how the normal, conventional *internal* lymph nodes function. So, this section addresses conventional lymph nodes, and briefly describes the main types of immune cells that get together, and interact with each other, in conventional lymph nodes.

When viewed from one perspective, a mammalian immune system must be regarded as a single complete system, which works because all of its components work together, in mutually supportive ways.

However, that is only one perspective; and, just as any complicated three-dimensional or living thing can be better understood by looking at it from two or more different angles, immune system should also be regarded as having very different aspects, which look different, from different angles. Accordingly, in addition to being a single coordinated system, an immune system should also be seen as a scattered array of essentially independent and autonomous outposts, each of which must be more-or-less self-contained, and able to protect and defend itself and its surroundings. In a very real sense, the "lymph nodes" in an animal can be regarded as analogous to the types of forts that settlers and armies build, when they are trying to occupy some contested area, and take it away from its previous (and hostile) owners. To provide an

adequate defense, a fort or military base in a hostile or frontier area must be equipped, supplied, and "manned" in ways that will allow it to immediately begin fighting back, as soon as an attack begins, without having to wait for instructions, weapons, ammunition, or additional troops from elsewhere.

In an analogous manner, the lymph nodes, which are distributed around numerous locations in the body, function as storage depots, armories, bunkhalls, and command posts, for all of the types of immune cells which will be needed to enable that lymph node – acting immediately and autonomously, without having to wait for anything from any "central headquarters" – to rapidly activate (and provide the necessary supplies and provisions for) all of the types of cells and molecules that might be needed to fight off an infection or invasion.

As a brief introduction, there are four different types of immune cells that play crucial roles in forming antibodies that can help fight off infections by pathogens. Those four types are:

1. DENDRITIC CELLS

The term "dendritic cells" was created by Ralph Steinman, who discovered them in 1973. He won a Nobel Prize for that discovery, but not until almost 40 years later, in 2011. As a brief aside, Steinman was, and remains, the only person who has ever been awarded a Nobel Prize posthumously. He died (of cancer) three days before the award was announced, but the awarding committee did not know that. When they learned – after announcing his prize – that he had recently died, they decided to grant the award despite his death, partly as the dignified thing to do, and partly because – by then – everyone who had reviewed the science realized how crucially important dendritic cells (as first discovered and recognized by Steinman) are, in any effort to figure out how the immune system actually responds to invaders.

The term "dendrite" arises from the Greek root for "branching", or "tree-like". When seen under a light microscope, a dendritic cell has numerous small and thin cellular projections, extending outwardly in multiple directions from the main body of the cell, with a generally "radial" appearance that looks similar to the branches of a tree, rather than looking like a batch of limp noodles.

However, "dendritic cells" turned out to be an unfortunate name, for several reasons. One problem was that other types of cells (especially neurons) also contain branch-like projections that are also called "dendrites"; and, that overlap and conflict apparently blocked or prevented the emergence of a single-word name (such as dendricytes, or dendrocytes). Accordingly, the term "dendritic cells" became and remains the standard term, and whenever a physician or researcher hears that phrase, they have to do a quick but distracting mental check to ask, "Are we talking about neurons, or immune cells, at this moment in time, and in this context?"

In addition, subsequent research (after Steinman had already assigned an unfortunate name to them) revealed that their "dendrites" are not actually tubular, and do not resemble the branches of trees. Instead, they have substantial width and flatness, and are more similar in shape to petals on a flower (or leaves on a "succulent" plant), than to branches on a tree. The wider, flatter shape provides the projections with more surface area, which is needed for large numbers

of surface receptors, and for surface-mediated activities that are carried out by the cells.

When first formed, inside blood marrow, and while they travel in circulating blood, dendritic cells (and macrophages, also) are called “monocytes”. At some point in time, special adhesion molecules on the surface of a monocyte will grip the inside of a blood vessel, and the monocyte cell will shift into a fluidized, amoeba-like structure which allows it to pass through a gap it will then create between the cells of blood vessel wall. It thereby leaves the blood vessel, and enters the watery fluid that forms roughly 1/6 of the volume of most soft tissues (called “lymph” herein, but not all experts use that term in that manner, as discussed below).

When a monocyte emerges from a blood vessel and reaches the lymph fluid, it is called an “immature” dendritic cell. Although scientists are not certain, it is possible that all, or possibly just some, monocytes are capable of transforming into either macrophages or dendritic cells, when they first emerge from a blood vessel and enter the lymph fluid, and the pathway which will determine whether a specific monocyte will become a macrophage, or a dendritic cell, depends on what it encounters during its maturation process.

Although dendritic cells can be divided into several categories (which have been given names such as “plasmacytoid DCs”, depending on factors such as which particular cytokine molecules they carry or release), the largest and most populous category is called “conventional” dendritic cells, and all comments herein refer and relate to that category.

Most immature dendritic cells initially travel to the soft tissues in the shallow layers directly beneath an epidermal surface (i.e., “dry skin”) or mucous membrane. They do so because that is where their services most likely will be needed, to respond to pathogens which have managed to penetrate the outer defenses of an animal body and enter the tissues where the nutrients are. As an illustration of that fact, when “monkeypox” viruses began spreading rapidly, in 2022, hard on the heels of the COVID epidemic, health authorities began struggling to make a tight and limited supply of the vaccines, available to all who wanted them. To stretch their supplies, they began injecting only a half-dose, in a shallow “sub-cutaneous” layer just below the skin, and the people doing the injections distributed that “bolus” of liquid across a wider area of soft tissue, by gradually withdrawing the needle, as they ejected the liquid from the tip of the needle. Those vaccines were every bit as effective as conventional injections using twice the dosage, since the conventional injections were driven ¾ inch deep, beneath the skin, by jabbing the same ¾ inch needles as deep as they could go. That deeper layer is not where most of “the action” is; and, since the immune system evolved in ways that made it as effective as possible, that deeper zone is not where most “mobile” immune cells live, and travel, and patrol, in much higher numbers.

If and when an immature dendritic cell encounters a pathogen, the dendritic cell will not simply ingest the pathogen and begin processing it. Instead, the cell will hold the pathogen on its surface for a sustained period of time, while various “pathogen processing receptors” on the surfaces of the dendrites (i.e., the appendages which extend out from the body of the cell) will interact with proteins and other molecules on the surface of the pathogen. Any reader who tries to learn more about those “pathogen processing receptors” will encounter phrases and acronyms

such as “toll-like” receptors, CR and CCR receptors, mannose receptors, lectin-like receptors, dectin receptors, DC-SIGN receptors, DEC receptors, etc. No one (other than full-time immunology specialists) needs to go digging into those labels or acronyms; it is enough to know that lots of different kinds of pathogen receptors exist, and it was a remarkable feat that highly specialized “genius caliber” cells managed to evolve with extra-ordinarily large numbers of different receptor types, all on the surface of a single cell, and with all those different receptors able (unless something goes wrong) to function in harmony, and in parallel, in thousands of generations of such cells, over spans of multiple decades for at least some types of animals.

At some point, if enough signals are detected by an immature dendritic cell to activate the maturation process, the dendritic cell will shift into full maturation mode, and will commence a process called “antigen presentation”. That process can take either of two different pathways, depending on whether the dendritic cell commits to either: (i) antibody formation; or, (ii) killer T cell activation, as the end result. Regardless of which of the two pathways a dendritic cell will “commit” to, the processing of either a pathogen, or a vaccine particle, by a dendritic cell, presumably will include the following steps:

(i) taking in the pathogen, via phagocytosis (discussed above), inside a membrane enclosure called a phagosome;

(ii) partially digesting and breaking apart the pathogen, in ways that create “chunks” of proteins that previously were positioned on the surface of the pathogen (or vaccine particle);

(iii) mounting those semi-digested protein “chunks” onto a specialized type of “mounting plaque” protein, called an MHC protein (the acronym for “major histocompatibility complex”);

(iv) moving those “mounted chunks” of semi-digested proteins (and the MHC “mounting plaque” proteins) out to the surface of the dendritic cell.

The qualifying word “presumably” was included, just above that list, because, in at least some cases, MHC surface proteins may play an active role in binding to a pathogen, via a relatively short peptide sequence (usually involving 20 or fewer amino acids) on the particle which appears to have an alien “non-self” sequence, and then enabling a dendritic cell to begin cutting and trimming off the “flanking” portions of that protein which did not bind to the MHC protein.

Inside the body, that multi-step process is performed while the dendritic cell is traveling toward a lymph node; and, the fact that a dendritic cell, right when it matures, will suddenly leave the quiet shelter of a docking site on the back side of an M cell, and begin traveling toward a lymph node, is no mere coincidence. That process . . .

[THIS PASSAGE WAS DELETED, SINCE IT VEERS A BIT TOO CLOSE FOR COMFORT, TO THE INVENTION ITSELF]

As a brief aside, numerous articles by experts refer to “maturing” rather than “mature” dendritic cells. It is not clear, in most such articles, just when, where, and how that “maturing” process becomes finally complete, so that a dendritic cell can and should be called fully

“mature”. This application assumes that a dendritic cell becomes fully “mature” once it has reached the germinal center of a lymph node, and has handed off (to B and T cells) the semi-digested “chunks” of protein it processed while it was traveling, and is free to leave that lymph node, and go wherever its pathway leads it next. However, for simplicity, the term “mature” as used herein indicates that a dendritic cell has received an apparently pathogenic particle, has used its surface receptors to analyze that particle, has fully “committed” to the process of antigen processing and presentation, and has translocated the receptors to its outer surfaces, which will enable and motivate it to travel toward a germinal center in a lymph node.

When an activated and “mature” dendritic cell reaches a lymph node, it will be carrying some number of “chunks” of protein, created by a controlled process of semi-digesting the particle (which usually will be either a pathogenic microbe, or a vaccine particle) which caused it to “mature” and shift into an “antigen-presenting” mode. Those “chunks” of protein usually are about 10 to about 12 amino acids long, and the dendritic cell will place them on special “mounting plaque” proteins (called MHC proteins, discussed below). That “mounting” process creates a set of signals, for the B and T cells which will take over the process of antibody formation, inside the lymph node, after a dendritic cell (or macrophage cell) “presents” its semi-digested protein chunk(s) to the B and T cells, which are discussed below. Alternately, as mentioned above, if a foreign particle binds to an MHC surface protein on a dendritic cell, the dendritic cell might simply begin cutting off the flanking portions of the particle’s protein, while leaving intact the peptide portion which became bound to the MHC protein, which will be presented to the B and T cells.

When a dendritic cell (or a macrophage cell, as described below) encounters an apparently foreign particle, it can respond by “committing” to either of two very different pathways. If the particle does not seem especially dangerous or important, it can simply ingest the particle, and break it all the way down to its building blocks (which are secreted by the immune cell, so that other cells can use them as nutrients), without further ado, disruption, or demands on its time and efforts. In the other possible pathway, a dendritic cell will “commit” to a much longer, more elaborate, multi-step, time-consuming process called “antigen presentation”.

Accordingly, one of the goals and requirements of any effective conventional vaccine formulation is to create some combination of particles, and additive compounds called “adjuvants” (discussed below), which will encourage and provoke the immune cells which encounter those particles, to commit to antigen presentation.

It is worth a digression to comment on words such as “choice” and “commit”, when referring to what immune cells do, when they are at a point where they might take either of two different pathways. It is proper and accepted usage to state that a macrophage or dendritic cell has “committed” to taking either one pathway, or the other pathway. However, one should never use words such as “choose” or “decide” or “select”, to describe that process. Experts will pounce on such ill-chosen words, to make a point of pointing out that a cell does not have a brain, and cannot think, and therefore cannot “choose”, “decide”, or “select” anything.

In normal usage, when applied to people, the words “commit” and “commitment” usually imply a *higher* level of dedication, resolve, courage, commitment, and complexity, than words such as “choose” or “decide,” which can be performed immediately. A person can make a “decision” quickly, and on trivial matters, such as by simply choosing what to eat, what TV show to watch, etc. By contrast, among humans, a “commitment” (such as a commitment to study harder and get better grades, or a commitment to at least try to be nicer to other people) suggests that a decision has been coupled with, and harnessed to, some significant level of resolve, determination, and self-discipline, which will be required to carry out that decision over a prolonged span of time.

Among cells, the implications of those same words point in the exact opposite direction. A cell can be evaluated, to determine which of two candidate pathways it took, and the phrase, “The cell committed to this pathway, rather than that pathway,” is entirely appropriate. However, a phrase such as, “The cell chose (or decided) to follow THAT pathway,” suggests, improperly, that cells can do something which resembles conscious, analytical thought, and which somehow allows them to consider and evaluate different options, and then make a decision. Any “expert” who hears or reads such a statement usually will regard it (conditions permitting) as an opening, opportunity, and invitation to display his/her superior knowledge and understanding, by pointing out that mere cells cannot think, cannot “choose”, and cannot “decide”. They can indeed commit, yes; but, they did not “choose” whatever it was that they “committed” to. Instead, their receptor molecules simply responded to a set of stimuli.

Returning to the main topic, if a dendritic cell commits to a process of antibody formation, it will place the semi-digested chunks of protein on “MHC-2” mounting plaques; alternately, if it commits to a process of activating killer T cells, it will place the semi-digested chunks of protein on “MHC-1” mounting plaques. No one other than an expert is expected to memorize and remember which type of MHC protein is involved, in which process; however, if someone wants a “mnemonic trick” to help them remember, they can use the following:

(1) an antibody will have *TWO* arms which can latch onto pathogens; and, if an antibody response is being triggered, a dendritic cell will use an MHC-*TWO* protein;

(2) by contrast, it only takes ONE activated killer T cell to gobble up and kill pathogen-infected cells; and, if a T cell activation response is being triggered, a dendritic cell will use an MHC-*ONE* protein.

Once a dendritic cell has reached a lymph node, and has completed the process of interacting with B cells and “helper T cells” (in ways that presumably involve “handing off” any MHC-mounted chunks of semi-digested protein to either a B cell or a T cell), a presumption arises that the dendritic cell has finished its work, and the B and T cells will take over. It is not known (at least, not to the Inventor herein, although experts may know the answers) whether, how often, or under what circumstances, a dendritic cell will be digested, as part of that process, or whether it will simply leave the lymph node and go elsewhere, where it might be needed, and might be able to carry out the same process, with a completely different apparently pathogenic process. A presumption has arisen that when a dendritic cell matures, in response to encountering

a pathogen, it will devote the rest of its life to fighting that particular pathogen, rather than cycling back through the same process with a different pathogen. However, that presumption may have arisen, not from direct observations, but from the fact that new dendritic cells (which will be able to interact with other, different pathogens) are constantly being made at fairly high rates. Liu 2007, which reported a study of mice, estimated that about 4,000 new dendritic cells were created by the immune system, every hour, in a mouse. If scaled up to the weight of an adult human (based on an average mouse weight of 0.8 ounces, and an average adult human weight of 180 pounds, which is 3,600 times heavier than a mouse), that would suggest that adult humans create somewhere in the neighborhood of about 14 million new dendritic cells, every hour.

Dendritic cells also are known to play crucial roles in activities that are called "tolerogenic", since they help promote tolerance, in the immune system, to surface proteins and other molecules that are found in each individual host animal. Those activities include, for example, (i) "sampling" (also called "nibbling at") molecules on the surfaces of "self" cells; and, (ii) actively killing off T cells that otherwise might cause auto-immune problems.

In summary, dendritic cells play absolutely crucial roles, in all vertebrate immune systems; and, it is something of a testament to their subtlety and sophistication, that scientists could not even begin to figure out what they were, what they did, or how they did it, until the 1970s. Today, there are thousands of "review articles" in which top-level researchers try to extract organizing patterns and principles from among the huge amounts of data that have been gathered, on dendritic cells, by other researchers. Entire books also have been written about them (e.g., Lotze and Thomson, *Dendritic Cells: Biology and Clinical Applications* (2nd ed., Academic Press, 2001), and Welles, *Dendritic Cells: Types, Life Cycles, and Biological Functions* (Nova Biomedical, 2010)). A three-volume collection attempted to gather together the best papers that had tried to explain what was known about them as of 2006 (Lutz and Romani, eds., *Handbook of Dendritic Cells: Biology, Diseases and Therapies* (Wiley VCH, 2006), and an entire laboratory manual is devoted to the best tools and tricks researchers had been able to come up with (as of 2010) in their efforts to study dendritic cells (Naik, ed., *Dendritic Cell Protocols – Methods in Molecular Biology* (2nd ed. (2010), Springer Protocols).

2. B CELLS

B cells are the only cells which can make antibodies; or, stated in alternate terms, any cell which can indeed create antibodies, is called a B cell, regardless of what type of animal it is in.

As a brief aside, they were given that name, because the first-ever discovery of how B cells are created, involved a pouch-like tissue structure in chickens, known as the "bursa of Fabricius". "Bursa" is the Latin word for pouch, or purse, and Fabricius was the anatomist who first noticed and described it, in the 1600's. No one knew what it did; so, in the 1950s, a bird scientist named Bruce Glick, at Ohio State University, removed it from baby chicks, to see how it would affect their development. It had no apparent effects, so he gave some of his "bursectomized" chickens to a graduate student, Timothy Chang, who needed birds that could

produce antibodies against a mild and weak strain of *Salmonella*. However, most of the birds that were infected by the weak strain of *Salmonella* died, and even those that survived did not make any antibodies, at all. When Chang and Glick began to study why, they discovered that the bursa, in birds, created and then released a crucial set of immature immune cells, which could then mature into the cells that could create antibodies. Those cells were called "B cells" (from "bursa"), and that name is used for all types of animals that make antibodies, even those that do not have a bursa.

Mammals do not have the types of "bursae" which birds use to make B cells (or B cell precursors). However, confusion over terms can arise, since mammals have totally different types of bursa tissues, which are pouches of tissue where "synovial fluid" (which lubricates "articulating" joints, such as knees, shoulders, hips, wrists, fingers, etc.) is formed, before moving into the sliding contact area between two cartilage segments, in a joint. Humans have about 150 different bursal pouches, all located near joints, and the arthritic condition called "bursitis" occurs when a bursal pouch becomes swollen, inflamed, and painful. However, those pouches have nothing to do with "B cells" of the immune system; instead, mammals make B cells from precursor cells that are made in bone marrow.

B cells make the "variable fragments" of antibodies (i.e., the active binding portions on the two "arms" of the classic Y-shaped structure) by shuffling around segments of DNA, within a carefully-limited "hyper-variable" region of a specific chromosome. That ability is unique to B cells; no other type of cell can do it. The process which is used, inside lymph nodes, to create various "first-round candidate antibodies" – and to then choose which B cells made "first-round candidates" which were good enough to allow those particular B cells to move on to a second (and then a third) round of "selection" – is a complex process, which takes well over a week (and often close to three weeks) to play out.

When B cells begin trying to make new antibodies, with never-before-created variable (Fv) segments that will bind to a chunk of antigenic protein which has been presented to them, the initial antibodies will be a specialized type of antibody, called an IgM antibody. These are, in effect, small "trial size" antibodies, which can be made more rapidly and easily than full-size IgG antibodies. The B cells which create those IgM trial-sized antibodies will "offer" those new IgM antibodies to "helper T cells" which have incorporated, adopted, or otherwise responded to the antigenic protein sequence which were brought to the lymph node by a dendritic cell (or macrophage, as discussed below). Those B cells which offer up the "most promising" IgM candidates (i.e., those which can bind with some respectable level of strength and selectivity, to the antigen sequence), are stimulated by "cytokine" molecules (discussed below) from one or more helper T cells, and will reproduce and begin forming a "second round" of IgM antibody candidates, which will be variants of the first candidates. Those "second round IgM contenders" will then go through additional rounds of selection, involving the T cells. By contrast, B cells which produce poorly-performing IgM candidates are not stimulated, and commence a process of "programmed cell death", which gets them out of the way.

After several cycles of that type of selection – with each cycle usually leading to better-

binding antibody candidates – a T cell which detects a potently-binding IgM antibody, on a particular B cell, will shift into a "final approval" signaling mode. The B cell(s) which are activated by those signals will begin reproducing, to make more copies of active B cells which will begin making full-sized and complete IgG antibodies, instead of the smaller "trial-sized" IgM antibodies.

That process – which can be likened to an elaborate dance, where the losing suitors will be killed – is a sophisticated method of selection and development, and it took decades for scientists to figure it out. Videos which visually depict and explain those processes include youtube.com/watch?v=GXVLdbkRkhw (Dr. John Looney, T Cell Activation and Control) and youtube.com/watch?v=TMnkihN6zVM (Armando Hasudungan, MHC II Processing).

Even under the best of conditions – in a healthy young person who is not struggling with a serious disease – that multi-cycle process, in which several different types of immune cells must cooperate and work together, to figure out how to make good IgG (internal) antibodies to help fight off an infection, takes at least a week, at a minimum; and, it often requires more than two weeks, especially among elderly people, immuno-compromised people, and people whose immune systems are already laboring to fight off some other disease. That is why public health agencies require everyone to assume that no vaccine can offer any genuine protection, until about 3 weeks after the vaccine has been injected; and, it is why research scientists wait for 3 weeks, as a standard waiting period, after a test vaccine has been injected, before they measure antibody responses in any lab animals the vaccines were injected into.

It also helps to explain why, in 2021-22, so many thousands of people who refused to get vaccinated against coronavirus until *after* they became infected, ended up driving thousands of highly qualified doctors and nurses out of health care, even though those same doctors and nurses had worked heroically, and with extraordinary dedication, perseverance, and grit, despite mortal danger to themselves, throughout the entire first major stage of the pandemic, until the first vaccines became available.

Those doctors and nurses acted heroically, before the vaccines became available, because they knew they were trying to help innocent victims. However, after vaccines became available – and, after it became clear that those vaccines were truly and remarkably effective in preventing severe cases, the need for hospital care, and deaths – it became intensely depressing, and demoralizing, when those already exhausted and over-stressed doctors and nurses realized that, except for the children who needed help, they were no longer trying to help innocent victims. Instead, they were being asked to continue subjecting themselves to endlessly more years of horrible stress and hardship, to try to treat large numbers of intensely angry, embittered, unpleasant people who had willfully and deliberately abused and ignored every opportunity to protect themselves, and who then – only *after* they became terribly sick, and desperate – began demanding the vaccines.

For the most part, the doctors and nurses who were being hammered by those demands for vaccines, by people who were already sick, usually said no (or at least tried to say no), the first few times those mal-informed, misguided, horribly sick people demanded the vaccines,

because they knew, quite well, that it was far too late for any vaccines to do any good, by then. The doctors and nurses knew, all too well, that any vaccines which were administered at that stage would be much more likely to clutter up, interfere with, and distract from the already-intense efforts of the actively-involved cells in the immune system, to create good antibodies against the coronaviruses.

However, in most cases, any attempt to say no, and to explain why they were saying no, only triggered those patients and their families into becoming even angrier, and even more aggressive, insulting, and abusive. So, at varying points, after the vaccines had been readily available for months, literally thousands of very good doctors and nurses ended up quitting, and leaving the practice of medicine, because the adults who kept showing up at hospitals, desperately sick and pathologically angry, were those who had consciously, knowingly, deliberately refused to get vaccinated. Regardless of what types of public comments those doctors and nurses made, to justify their decisions, one of the underlying and real reasons was a powerful sense of, "I've reached my limit, and trying to treat *patients like these* just is not worth it, any more."

One can only wonder whether, if more patients – and, more of the politicians who deliberately chose to try to inflame, aggravate, abuse, and exploit the divisions, polarization, and uncertainties among the populace – had known, a little better, how vaccines actually work, and why it takes more than a week for them to become effective, many of those problems could have been avoided. One can only wonder how much public funding could have been saved, and spent on other things, rather than on people who were desperately sick only because they had deliberately refused to get vaccinated. One can only wonder how many patients suffering from cancer – or numerous other diseases that kept getting worse, with every day, week, and month that passed without treatment – could have been diagnosed and treated earlier, better, and with less damage to those patients, and lower costs for the medical and insurance systems – if the innocent people suffering from those other types of problems had not been forced to wait, while their diseases keep getting worse, for weeks or even months, until after "the self-inflicted anti-vaxxer COVID crisis" surge finally began to taper off. And, one can only wonder how many of those doctors and nurses who quit – because they became fed up and discouraged from treating, not just sick, but sick, angry, and abusive anti-vaxxers who would not willingly listen to good medical advice, even in the face of a deadly epidemic – would still be helping people who truly need medical help, today.

3. T CELLS

T cells were given that name because, after they are initially formed (in immature form) within the bone marrow, they pass through a highly specialized piece of tissue called the thymus, which sits in a gray-area boundary zone, where it is something of a gland, and something of an organ (and which, therefore, is often called "a lymphoid organ"). It is located between the lungs, beneath the sternum (i.e., the wide and flat "breastbone" between the left and right ribs, in front of the lungs). And, despite their similar names, the thymus should not be confused with the

thyroid, a true gland, which is located higher, near the base of the neck, not covered by a bone.

T cells have extremely, extraordinarily complex surfaces. Anyone who has not seen a picture of one should go to the Wikipedia entry on “T cells”, look at the first picture (which, although colorized, is an actual photograph, taken by an electron microscope, rather than some fantasy drawing), and ponder, for a few moments, how any cell could be that beautiful, and why any cell would be that complicated.

When T cell precursors mature, they must commit to being either a “helper” T cell, or a “killer” T cell. Immature T cells which will become “killer T cells” usually do so in lymph nodes, where they undergo a process which will cause them to commit to destroying any cells they encounter which have a certain specific protein sequence, on their surfaces; and, it should be noted, they can be “trained” (or primed, activated, etc.) to kill cancer cells which contain unusual cancer-related proteins on their surfaces. After they mature into actual “killer T cells”, they leave the lymph node, and begin to travel (slowly, unless and until they are shifted into a “hurry up” mode by messenger molecules called chemokines and cytokines) through the clear extra-cellular liquid called lymph, in soft tissues. They are analogous to policemen who, instead of riding in a car, have been assigned to walk down sidewalks and alleyways, looking and listening for signs of trouble. If they spot trouble – such as cancer cell which has unusual proteins on its surface, or a cell which has become infected and has moved distress-signaling marker proteins to its surface – they will engulf it, kill it, digest it, and release its building blocks, as nutrients for cells in the vicinity. A single killer T cell can kill any number of “bad guy” cells, and microbes; and, it will act on the spot, quickly and decisively, rather than carrying something to a lymph node, so that other cells can analyze it and respond to whatever it is.

It was mentioned above that immature T cells become either “killer T cells” (also called “cytotoxic T cells”), or “helper T cells”. While some “helper T cells” remain in lymph nodes, where they play crucial roles in helping to regulate and assist in antibody formation (and in anything else which happens in a lymph node), other helper T cells leave the lymph nodes, and go out “wandering”. They offer assistance to any dendritic cells, B cells, or macrophages they encounter as they pass through soft tissues; and, some of them end up settling into the surface-mounted lymph nodes called MALT patches, described above. Those that settle into MALT patches will function in much the same way they function in internal lymph nodes. They will interact with dendritic cells (which will show up with new challenges, handed off to them by M cells in MALT patches) and B cells (which will respond to those new challenges by creating new types of antibodies), in ways that will lead to the creation of IgA antibody dimers (rather than stand-alone Y-shaped internal IgG antibodies) which will be secreted into saliva and other mucosal fluids.

So long as a reader is generally aware that “helper T cells” play important roles inside lymph nodes (including MALT patches), and interact with B cells that are trying to create new antibodies to help fight off some new invader, it is not necessary for anyone to know more about T cells, to understand the current invention. Therefore, they are not discussed in any more detail herein.

4. MACROPHAGE CELLS

Macrophages initially evolved as part of what can be called "innate" immune systems, in very early animals, during the development of early types of animal life, on earth.

The *innate* immune system, in any type of animal, is "hard wired" and works rapidly, without requiring any delay while waiting for antibody production (or T cell activation) to be completed. This is analogous to the way a nervous system enables rapid "reflex" responses, such as withdrawing a hand or paw immediately, if a hot surface is touched. Even very small non-mammalian animals (including insects, nematodes, etc.) have defender cells which help them fight off pathogens and parasites, in ways that qualify as "innate" immune responses. The more advanced types of immune systems, which did not arrive until later, were (and still are) called *adaptive* immune responses, if they involve multi-step sequences of actions that require coordination and cooperation between various different types of cells.

One of the first patterns used by early multi-cellular animals, to defend themselves against infections by microbes, was a simple analysis based on size. Cells in animals tend to average about 10 microns in diameter, while most bacteria and other "non-eukaryotic" cells (i.e., cells too small to have a nucleus, also called prokaryotic) average only about 1 micron in diameter. Therefore, size became a very useful differentiator, in animals with early defensive systems, between "self" versus "non-self" cells or particles.

With that as a starting point, evolution then began the process of discovering and refining numerous additional factors which animal cells could use to differentiate between "self" versus "non-self". Accordingly, some of the earliest defensive mechanisms, in multi-cellular animals made of eukaryotic cells, became: (i) endowing essentially all cells with an ability to engulf and pull in any particles smaller than the cells themselves, via the standard process of phagocytosis, and then having those phagosomes merge with "lysosomes", which became membrane-surrounded droplets with high levels of acidity and digestive enzymes inside them; and, (ii) gradually creating a class of specialized defender cells, which could roam about, and perform the phagosomal intake trick more rapidly and effectively than any of the stationary cells. Accordingly, macrophages (that term translates into "relatively large cells which can devour other cells") became key components of innate immune responses; they are mobile cells which can directly and rapidly engulf and swallow up pathogens, and kill and digest those invaders.

As the earliest forms of animal life evolved into larger and more complex forms, innate (primitive) immune systems were already functioning, by the time adaptive immune systems began to evolve. Therefore, adaptive immune systems evolved in ways that used components of the innate immune system as their starting point. As a result, macrophages (i.e., the mobile "innate defender" cells) gradually were given additional duties, including the killing, digestion, and recycling of cells that had become "senescent" and too old to function properly, as the process of "programmed cell death" called "apoptosis" gradually emerged, under the control of mitochondria.

Through unknowable numbers of small evolutionary increments, macrophages gradually

developed an ability to respond to various types of indicator and messenger molecules, and that led to an ability to actively communicate with other types of cells, in ways which helped create the "adaptive" immune systems that exist among higher animals today.

Accordingly, macrophages perform essentially the same types of roles that dendritic cells perform, in terms of being able to analyze not-previously-encountered pathogens, and break them down, while traveling to a lymph node, into semi-digested chunks of protein that will be presented to B cells and helper T cells, to get the process of antibody formation started. However, macrophages do not have nearly as many different analyzing receptors on their surfaces, and they spend most of their time cleaning up, digesting, and recycling debris, rather than analyzing and processing not-previously-known pathogens.

In summary, those four cell types – dendritic cells, B cells, helper T cells, and macrophages – are the four most important cell types which drive and control the process of antibody formation, in internal lymph nodes. All four of those cell types are mobile, and all four of them are present and active, not just in internal lymph nodes, but also in the surface-mounted lymph nodes on mucosal tissues. So, attention will turn, soon, to those surface-mounted mucosal lymph nodes, but before it does, a general comment ought to be considered.

A PLEA FOR TOLERANCE RE: ARGUABLE TERMS AND PHRASES

Because of a number of factors, and before diving deeper into more detailed aspects of mucosal tissues, cells, and vaccines, some general comments are deemed to be not just appropriate, but necessary, about problems that arise when scientific and medical terms are used inconsistently, by different people.

Examples of these problems arise from a number of specific terms that appear or are assumed to be precise, but which are used inconsistently, even by high-level scientific and medical experts; and, several examples are offered in the next section, below.

However, larger and more important issues have come to clutter, confuse, and bedevil even the most sincere attempts to communicate useful information, concerning vaccines. Unresolvable disputes (and a serious level of public distrust, or at least unease) over whether some types of vaccines may have caused (or aggravated) cases of autism, in children, already had been bubbling for decades, when the coronavirus pandemic suddenly arose, and killed more than a million Americans, with the absolute worst possible political timing, during an intensely divisive and even poisonous Presidential election (in 2020), in ways which guaranteed that people would become bitterly divided, angry, and hostile over whether they (or anyone else) should get vaccinated, when the vaccines finally became available (which did not occur until after the November 2020 election, leading to still more accusations).

As part of a conscious attempt to minimize the risks of getting mired down in politically motivated (or shaped) arguments, and recognizing in advance that anything written herein can be distorted and misrepresented by anyone who reads it and has their own agenda (of any sort), this patent application makes a serious and sincere attempt to describe this invention, using terms and analogies that attempt to bridge the large and severe gap between: (i) the language and terms that

scientific and medical experts regard as optimal, versus (ii) the language and terms that predominate among other groups, including but not limited to: (a) business and financial managers who are not scientific experts, but who must approve the types of funding that this research will need, if it is to progress beyond proof-of-concept studies in small animals; and, (b) journalists, internet mavens, trolls, and "influencers" who, in their own widely varying ways, will attempt to control or at least influence how these matters are described to the public.

Accordingly, this is a direct, explicit, and unapologetic request for tolerance, among people on both sides of any arguments or debates, when it comes to any terms, analogies, or other efforts used – herein, or by anyone else, at any time – as part of any effort to describe: (i) the components that will be incorporated into any final assembled vaccine particles designed as described herein, for mucosal administration; and/or, (ii) the processing steps that can be used to create such vaccine particles.

Beyond that, it is the direct, explicit, and sincere hope, of both the Inventor herein, and the patent attorney who drafted this application, that any experts who have received enough education, training, and exposure to enable them to grasp and understand the teachings herein – more rapidly, insightfully, and/or comprehensively than non-experts – should recognize, accept, and even take pride in shouldering the special obligation that experts should willingly accept, and bear, to do everything they can – with the assets and resources they have, and the responsibilities they have been entrusted with – to help create and promote useful, productive, and insightful discussions and debates, rather than causing or allowing such discussions and debates to degenerate into malice and anger, or by acting in condescending and insulting ways toward those with less (or differently-focused) expertise.

Regrettably, some scientists will seize on any usage of a technical term which they choose to regard as incorrect, or even just suboptimal, as an opportunity to attack, criticize, belittle, and undercut anyone who, in their opinion (or at least in their hopes), does not understand some particular subject as well as they (the experts) do. That type of condescension and belittling is not merely unhelpful, it is actively counter-productive to good science, and it contributes (in negative and even toxic ways) to the types of anger and antagonism that have contributed, not just to the arguments that are occurring over vaccines, but to larger breakdowns in the relationships between scientists versus other segments of society, in larger matters, including matters of truly urgent worldwide importance (such as bitter, nasty, and unproductive scientific disputes and arguments over climate change and greenhouse gases; far too many politicians have used those disputes and arguments as "cover", to justify their failures and refusals to take reasonable and appropriate steps to try to at least mitigate the huge and horrible problems that are approaching).

One must also bear in mind that dictionary definitions are obliged and compelled to be condensed, compact, and concise, rather than adaptable, flexible, and able to anticipate questions that often could not even be asked when those definitions were written. A typical definition in a dictionary is limited to somewhere between 2 and 4 lines. By contrast, when a patent attorney writing a patent application must try to explain, in detail, how some important term should be

interpreted and applied, not just when written, but in light of developments and discoveries that may occur during the following 20 years (which is the "lifetime" of a patent), a thoughtful, insightful, and diligent attempt usually requires at least a full page, and often several pages.

Furthermore, if five different dictionaries are consulted, to see how they each define a single, specific, exact word having a complex meaning, those five dictionaries are almost certain to have five different phrasings. If some well-funded company decides to create a team of lawyers and scientists, and assign that team the task of finding borderline, shades-of-gray, play-any-angles cases and examples of how those five different phrasings might be construed and interpreted differently – with a $20,000 cash award for every example they can find, and every argument they can make with a facial expression of sincerity (with unlimited practice sessions allowed), regardless of the actual truth or sincerity of that claim, in an effort to deliberately confuse and befuddle at least one person on a jury – any skilled team like that could find numerous clever ways to grab multiple $20,000 rewards, for that kind of effort.

One also must bear in mind that the relevant regulatory agencies, in more than 150 different countries and regional organizations around the world – including the U.S. Food and Drug Administration, the European Medicines Agency, and corresponding agencies in more than a hundred other nations – each have a sovereign right to adopt and then enforce some particular meaning, for any term that might be open for analysis or argument. And, when such terms are being debated, adopted, and used – either in statutes passed by elected bodies, or in regulations issued by agencies – those terms and phrases can be, and often are, affected (and in some cases deliberately distorted) by lobbyists working for companies and trade organizations which often promote needlessly complex and hyper-detailed definitions. As a rule of thumb, the more complicated and cluttered a regulation is, the more opportunities it offers, to companies affected by it, to play games with (and create arguments about) its meaning; and, those efforts, by lobbyists, are always and predictably designed and crafted to advance the interests of whoever is paying the lobbyists (rather than the interests of the mass of citizens, taxpayers, and society in general, or the goals of good science, and good research).

It also is a sad fact that many so-called "experts" (including depressing numbers of semi- and pseudo-experts, and outright charlatans and con-men) can and do find ways to undermine good science, if paid to do so by (for example) industry groups that want to continue selling harmful and/or dangerous (but profitable) products, or that prefer to ignore and belittle the scientific warnings about risks and dangers. The book, *Merchants of Doubt* (Oreskes and Conway, 2011), and the documentary film of the same name, offer a number of disturbing and regrettable pseudo- and anti-science examples of such behavior, by people claiming to be scientists and experts. If a person is being paid to deliberately inject confusion, uncertainty, and excuses for inaction, into public, political, and regulatory debates, then any terms and phrases which even might be defined and/or used in differing and inconsistent ways, will always, always offer opportunities to inject confusion, uncertainty, and excuses for inaction into such disputes.

Accordingly, in dealing with any matters addressed in this application, the request herein is for experts, and aspiring experts, to adopt – or at least try to reach and stretch in the direction

of – tolerant, compassionate, and accepting attitudes toward the use of laymen's and/or less-than-ideal descriptive terms, to describe any processes, components, or compositions discussed herein. If any experts believe that better terms, better phrases, better descriptions, and better explanations can help advance the science of better vaccines, then certainly, they should try to help create progress in that direction. However, that does not justify anyone in attacking, belittling, or mocking anyone else who makes a valid effort to understand the discussions and debates, or to describe any teachings herein, or any issues raised by these teachings, in ways that at least attempt to broaden and expand the levels of understanding among non-experts. An attitude of tolerance, and a willingness to genuinely and actually help, will be far more useful and beneficial than efforts to belittle and attack opponents, rivals, or even just "outsiders".

The matters discussed herein, about a new class of mucosal vaccines which may be able to substantially advance the level of art and science in that field, are of major importance to the health of the general public. Indeed, in view of the thousands of warnings that have been given, by true and genuine experts, about antibiotic-resistant microbes, the spread of tropical diseases into temperate regions, and pandemics (including the coronavirus pandemic which started in 2019/2020, and which continues to cause major problems, and which later expanded into a "tripledemic" that slammed American hospitals with a huge surge of cases that also included influenza viruses and "respiratory syncytial viruses" (RSV) as well), the question of whether vaccines having these new designs can be developed and commercialized, in time to provide large-scale actual benefits to society, may become vitally important life-and-death matters, for millions and possibly even billions of people. And, it also bears noting that many of the future vaccines described herein are likely to be paid for, some day, by taxpayers, in programs that must be acceptable to politicians.

Accordingly, these topics should be open for discussion by the public, the press, political leaders, and others, in tolerant and encouraging language and terms, without condescending, aggressive, officious-sounding demands for expert (or even hyper-expert) levels of precision. Any experts should at least try – as part of the burdens and responsibilities they took on, when they began wanting to be recognized and respected as true experts – to patiently strap on an extra layer or two of insulation and tolerance, when dealing with efforts by the public, the press, non-experts, and others, to discuss, debate, and try to actually understand these matters.

TERMS USED INCONSISTENTLY, EVEN BY EXPERTS

In an effort to illustrate and nail down the points made in the preceding section, this section sets out several specific examples where even experts use terms and phrases which are relevant to this invention, inconsistently.

As a starting example, the term "lymph" is used differently, by different experts. Some experts use the term to include any of the clear, watery, slow-moving extra-cellular (or "interstitial") fluids that are found outside of cells, in soft tissues. However, the classic and long-used definition of "lymph" says that such fluids do not qualify as lymph, *unless and until* they actually enter a lymphatic draining vessel (aka "channel").

The broader interpretation – which does not require those clear watery fluids to reach a lymphatic drainage channel, before they can properly be called "lymph" – is preferred herein, for two reasons. First, there is no other simple, convenient, straight-forward, and well-known term for such fluids; and more complex phrases (such as "extra-cellular fluids") are confusingly cluttered and polysyllabic, and require a listener or reader to pause, for several awkward moments, and try to parse any such phrase, to try to make sure they understand it accurately. Secondly: any such system of drainage channels which serve the surface-mounted lymph nodes that are called MALT (and NALT, GALT, etc.) patches, will inevitably be different from the drainage channels that are deep inside the body. A dendritic cell which is waiting for delivery of a pathogen, in the docking site behind an M cell in a MALT patch, is not in a drainage channel; instead, it is already in a specialized type of lymph node. So, a question arises: should the clear watery fluid located beneath the surface of a MALT patch be called "lymph", or not? And, if so, then exactly how deep, and how far, below the surface, should that definition extend? Can it be expressed in an exact number of microns, or cell layers?

Because of the second factor, it is hoped that medical schools, textbooks, researchers, and physicians will gradually shift to and adopt the broader definition of *lymph*, along the lines of, "the clear fluids that *lymphatic* cells dwell in, and travel through, in soft tissues."

Another example of an unclear, ambiguous, and arguable term, which is directly relevant to vaccines, is "adjuvant". As a general definition (discussed in more detail below) adjuvants are things that are added to vaccine formulations, to make them more effective and potent. However, because some things fall within "gray zones", and are neither black nor white, any number of arguable and borderline fact patterns arise, when people (even experts) try to figure out exactly what is part of a vaccine formulation, versus what is an additive. As an example that is relevant to the vaccines herein, suppose someone creates vaccine particles, by starting with an inert core particle, and then attaching various antigens to that inert core particle. The question will then arise: is the core particle, itself, an "adjuvant"? And, supposed someone adds an M cell targeting peptide, to a vaccine particle, as will be described below; is that transport peptide part of the vaccine particle, or is it an adjuvant, which has been added to a vaccine particle? Experts can argue for either answer, to either of those two points.

A third example of a term which is used differently, by different experts, is a term which most people regard as simply normal, rather than scientific; however, it easily can be turned into sources of confusion and disputes, if people with adverse motivations wish to entangle and distract from a useful analysis. Those who wish to do so can label the process of attaching additional molecules, to the surfaces of particles, as a "coating" operation, since all of the added molecules will indeed be affixed to the exposed outside surfaces of the particles. That is enough to satisfy one normal and sensible definition of "coating".

However, others can assert, if they choose, that the terms "coat" or "coating" are inappropriate, inaccurate, and misleading when used in that setting, if only a limited number of additional molecules are attached to the surface of a particle, if that limited number of added molecules falls far short of creating the type of consistent outer layer which some people regard

as being required, before something truly “coats” the surface of a particle. If someone sticks a hundred stick-pins into a medium-sized corkboard, those stick-pins will not “coat” that corkboard, under the normal meaning of the word “coat” in that setting.

The overlap-and-conflict between the terms “proteins” and “polypeptides” also merits attention, since those terms are used inconsistently, even by experts, largely because of how they evolved and emerged into current use, over time.

In all life on this planet, 20 specific types of “primary amino acids” are “strung together”, in specific sequences, to form proteins. The type of chemical bond that is formed between any two adjacent amino acid “residues” (as they are called, once they have become part of a larger molecule) in a protein uses a “peptide” bond to connect the amino acids to each other in a stable and consistent way; this type of bond always connects a positively charged amino group, at one end of an amino acid, to a negatively charged carboxylic acid group at the other end; and, this arrangement causes each of the 20 different primary amino acids to contribute its particular type of “side group” to the main “backbone” contributed by each different amino acid. This forms a long and complex molecule which will have both a shape, and a function, because of the selection and sequence of the 20 different side chains that are attached to the consistent peptide-bonded backbone.

When multiple amino acids are coupled together, via “peptide” bonds, the resulting molecule is, by definition, a “polypeptide”. Therefore, any “protein” has a molecular structure which necessarily and directly falls within the term, “polypeptide”.

However, the term “protein” already had been in use for centuries, before the atoms and molecules in peptide bonds were known or understood. It referred to a special and desirable class of foods or nutrients; the word itself, “protein”, arose from the Greek root “proto-”, which means first, or primary. For centuries, it referred to whatever was the critically important essential nutrient that was known to be plentiful in meats, and which also was present, but only in lower quantities, in other foods. It was not until the early 1900s, through the work of Emil Fischer and others, that scientists finally realized that “proteins” are made from amino acids, strung together via peptide bonds.

As the relationship between “proteins” and “polypeptides” became better known, and settled in among scientists, “protein” (when discussed in molecular rather than nutritional terms) came to imply, in the minds, words, and writings of many experts, “a complete polypeptide which is intact, and functioning.” And, since “protein” came to refer to “intact and functioning” polypeptides, the residual term “polypeptide” came to imply the other subset, i.e., it came to refer to strings or segments of amino acids which were smaller than – or which were obtained by breaking apart – a full-sized, complete, and fully-functioning protein.

However, that usage is not followed consistently, especially among non-experts. Instead, since the term “protein” is more familiar, and more easily remembered, spoken, and written than “polypeptide”, most non-experts call polypeptides “proteins”, regardless of whether they are intact or functional.

The more relaxed and common (non-expert) usage of “protein” is used herein, to include

any type of polypeptide which contains a plurality of amino acid residues, attached to each other via peptide bonds. This includes the types of semi-digested polypeptide "chunks" that immune cells create and use, to trigger and steer the processes of antibody formation, and T cell activation.

As a fourth and final example, if someone says or writes, "These viruses were killed, by using this process," some experts will use that "imprecise" usage as an opportunity to try to show how much more they know – i.e., how much more of an *expert* they are – than the person who used the word "kill". They will do so by pointing out that viruses are not even "alive", based on how experts define "life", and therefore, truly proper and precise terminology requires a poly-syllabic phrase, such as "rendered inactive" or "rendered nonviable".

However, regardless of whether scientific experts choose to recognize or try to resist any specific example, language (which includes "the meanings assigned to words") changes and adapts, over time, with "efficient, clear, and quickly-understandable communication" being one of the goals of at least some of those adaptations. Accordingly, phrases such as "We need to kill that rumor" have become entirely acceptable, even though rumors do not have metabolism, and are not "alive" by any biological definition; and, phrases such as, "Hey, stop, you're killing me," are also entirely reasonable, when used in joking and joshing situations, even though no one is being literally killed. Accordingly, one potentially useful standard that can (and arguably should) be applied, involves asking the question, "How would someone interpret this phrase, if they did not grow up in an English-speaking country, and if English is only a 'second language' for them?"

If that standard is applied, anyone should first spend some time, looking up and pondering the multiple different and potentially confusing and conflicting definitions of the word "rendered", in any good dictionary, before they begin arguing that the phrase "rendered nonviable" will make more and better sense, to someone who did not grow up speaking English, than the word "killed", when applied to viruses. If anyone wants to argue that the phrase, "We rendered those viruses nonviable, by doing XYZ," is a clearer, more helpful, more useful phrase than the simple and straight-forward, "We killed those viruses, by doing XYZ," – and, if they also believe that the billions of "English as a second language" (ESL) people around the world should be and will be able to figure out what that phrase means if they go to a dictionary, look up the word "rendered", and try to figure out which particular meaning was intended, in that particular use – they should be at least somewhat cognizant that the goal of "scientific precision" needs to be balanced against the goal of quick, efficient, and clear communication that can be understood by more people, all around the world.

And, by the way, even the very best experts use the term "virucide", to refer to things that render viruses nonviable. And "viru-cide" translates directly into "virus-killing".

The examples above should be sufficient to establish the basic point that even the top experts in the world don't always use terms and phrases consistently. Therefore, this is a plea for scientists and experts to show, and to actually use, tolerance, kindness, and encouragement, toward non-scientists and non-experts. Rather than seizing upon ambiguous and/or arguable

terms to try to belittle, contradict, criticize, or undercut someone who is trying to make a reasonable point or ask a reasonable question, scientists and experts should accept that their elevated position, as experts, brings with it an extra layer of responsibility, to try to actually and genuinely help others reach a better and higher level of understanding, of what the experts already know.

MUCOSAL DEFENSE COMPONENTS, AND "MALT PATCHES"

Driven by never-ending battles against invasive microbes, the mucosal components of immune systems evolved with an array of components, systems, and strategies, including:

1. A constant flow of sticky nasal secretions, which grabs hold of most particles (bacteria, viruses, airborne dust, etc.) and carries them downward, through the pharynx (i.e., the region at the back of the mouth, where the mouth, nasal cavity, and throat come together), and then down the esophagus, to the acidity of the stomach, which will kill most microbes.

2. The use of so-called "tight junctions" to join together adjacent epithelial cells on most of the surface areas inside the nasal cavity, pharynx, and mouth, to reduce the ability of at least some types of pathogens to target gaps, seams, or other weak spots in an exposed surface. These tight junctions are similar to the structures of capillary walls inside the brain and spinal cord, which form the so-called "blood-brain barrier", which keeps unwanted molecules away from neurons inside the brain and spinal cord. Those "tight junctions" are analogous to the classic battle formation used by foot soldiers, from the days of the Greeks up through the 1800s, in which every foot soldier on the front line of a formation held up a tall vertical shield, the width of a person, having straight vertical side edges, so that, when held next to each other, they formed a nearly solid (even if only temporary) wall.

Because of how those "tight junctions" function, in mucosal membranes, a fair statement can be made that, in almost all cases, the only way a pathogen can get into a mucous membrane, is by going through a cell, rather than trying to slip or wriggle through a gap between two adjacent cells. As a result, "MALT patches" (which are discussed in the next section) evolved to a point where they play absolutely crucial roles (which are far more important than would be suggested, by merely knowing how many of them there are, or what percentage of the mucosal area they occupy), in influencing and guiding how the mucosal immune system works, and how it responds to microbes.

3. Remarkably high numbers of antibody "dimers", having not just two but four "sticky arms" which can bind to pathogens, are secreted by certain types of mucosal cells directly into the nasal secretions and saliva. As mentioned in the Background summary, above, there are more than twice as many of those secreted IgA antibodies, floating in the thin layers of mucosal liquids outside the surfaces of those tissues, than are present in the entire remainder of the body.

4. And, finally, there are specialized types of tissue patches, mounted directly on the surfaces of every type of mucosal membrane, in any mammalian body. Those are the subject of the next subsection.

MALT, NALT, GALT, AND BALT PATCHES, and M CELLS

As mentioned above, the mucosal surfaces contain multiple surface-exposed tissue patches which act as a "first line of defense" against pathogenic microbes that are swallowed, or inhaled, or otherwise come into contact with the mucosal membranes. They can be regarded as surface-mounted lymph nodes (and they sometimes are called "secondary" lymph nodes), depending on how one defines the term "lymph node"; however, most scientists and physicians do not call them lymph nodes, because there are important differences between how the classic "internal" lymph nodes function, and how these surface-mounted "lymphoid tissues" function.

These special surface-mounted patches of active immune-system tissues are collectively called "mucosal-associated lymphoid tissue" (MALT) patches; and, they are divided into categories, depending on their location within the body. Those location-specific categories are referred to by the following acronyms:

NALT patches, which refers to *NASAL*-associated lymphoid tissue patches. The "N" letter in "NALT" also is sometimes referred to as "naso-*PHARYNGEAL*" tissue, to include the "pharynx", which is the region at the back of the mouth and top of the throat, where the mouth, nasal cavity, and throat come together. In humans, an entire cluster of NALT patches is arrayed in and around the pharynx, in a generally circular pattern called the "Waldeyer's ring". That non-continuous "ring" of multiple distinct patches includes the tonsils and adenoids (usually in pairs, on the left and right sides), as well as several other smaller NALT patches;

GALT patches, which refers to *GUT*-associated lymphoid tissue patches. These occur throughout the digestive tract, below the throat. Among doctors and scientists, they usually are called "Peyer's patches". It is worth mention that, in humans, a newborn baby already has roughly 100 of them. That number rises up to about 250, distributed along the entire length of the intestinal tract, as children grow into adulthood; and, it decreases, back down to about 100, after people pass the age of about 60; and,

BALT patches, which refers to *BRONCHIAL*-associated lymphoid tissue, in the lungs and windpipe. In most mammals, they do not appear inside the tiny "air sacs" inside the lungs; that is why they are called "bronchial" rather than "pulmonary".

Other MALT patches also are located in and around the eyes, and in the genitals, to help protect the mucosal membranes in those areas as well.

There is an extra-special subset of cells, mounted on the outer surfaces of all types of MALT patches. They are called "M cells" (some sources indicate that the "M" arose from "microfold", but others indicate it arose from "membrane"). These cells contain appendages (often referred to by terms such as "villi", which are thin enough to be hairlike, and usually incapable of independent motion) or "cilia" (which are thicker, comparable to the tails on sperm cells, and are often capable of controlled and sometimes coordinated motions), as well as ridges, ripples, and folds, all of which help increase the exposed surface areas of M cells, which increases their ability to contact and "sample" particles and molecules which are being carried past them.

There are two curious aspects of M cells, which merit a mention for each. One is that

some top-level immunologists assert that true “M cells” exist only in the intestines, as part of so-called “Peyer’s patches”, and that any equivalent or comparable cells, in the lungs, mouth, nasal cavity, and pharynx, are not actually “M cells”.

It is not known, to the Inventor herein, what the basis for that assertion is, and there are numerous publications (e.g., Brandtzaeg 2008, specifically entitled, “Terminology: nomenclature of mucosa-associated lymphoid tissue”) which directly refer to “M cells” in tonsils, adenoids, and other Waldeyer’s ring tissues. As used herein, the term “M cell” includes any cells on mucosal surfaces which are specifically adapted and equipped to scan particles that are “passing by”, and to perform a series of three crucial steps, when they detect a foreign particle that appears likely to be a pathogen. Those three steps, in sequence, are: (i) phagocytic intake, inside a bubble of membrane material (i.e., a phagosome); (ii) rapid “trans-cytosis” of the phagosome containing the particle, through the cell; and (iii) merger of the phagosomal membrane with the “basal” membrane of the M cell, leading to ejection of the unaltered (and “naked, once again”) particle into a location (called a “docking site” herein) where mobile immune cells (mainly dendritic cells) wait for such deliveries.

As an additional clarification, a cell does not qualify as an M cell, merely because it has various surface receptors which can trigger endocytosis of various types of particles. All cells have those types of receptors; for example, all cells which are classified as “anchorage dependent” (i.e., they belong to some sort of connective tissue, rather than being “free-floating” as with blood cells and mobile immune cells) have “integrin” receptors, which will grip – and in some cases, pull into the cell – various types of known “ligand” molecules. That “gripping activity” is used to help anchor cells to the extra-cellular matrix of collagen fibers which holds connective tissues together, and in other situations, integrins interact with signaling molecules. Two articles described below, cited as Staquicini 2021A and 2021B, identified a peptide sequence which drives cellular uptake by a specific type of integrin receptor, on the surfaces of lung epithelial cells. Those types of lung epithelial cells are not “M cells” as described above, and as recognized by immunologists.

The second curiosity is this: in articles such as Renfeng 2015, pictures taken by electron microscopes, of M cells in the intestines (where MALT patches are often called Peyer’s patches), appear to show that the M cells are recessed, and sunken, compared to the cell and membrane surfaces which surround them; and, they do not appear to have the types of finger-like “villi” that are found on M cells in the pharynx. Both that recessed condition, and the absence of villi on their surfaces, presumably would reduce, rather than enhance, their ability to “sample” things that are passing by, in liquids, foods, or air.

The reason for that apparent paradox is not known to the Inventor herein, but two possible explanations should be considered. The first is that the high levels of vacuum which must be created, before a scanning electron microscope photograph can be taken: (i) might pull off and detach any protruding appendages that were previously extending outward from the M cells; or, (ii) might induce more swelling and enlargement, in the surrounding epithelial cells, than occurs in M cells, which have internal structural components that evolved in ways that can

accelerate “trans-cytosis”, discussed below.

As another possible explanation, it may be that the M cell structures and arrangements, in intestines, evolved with different configurations than M cell structures in other locations, due to very different “pressurized contact” conditions. In the intestines, the soup-like liquified mixture that passes through the small intestines, and the initial portion of the large intestines as well (until sufficient water is removed, by the large intestines, to convert the soupy liquid into fecal matter), is under a form of constant pressure, due to a “peristaltic” pumping action inside the intestines, which is necessary to drive the food material which is being digested, through the long intestinal passageway. Accordingly, M cells in the intestines will have the liquid material (including any pathogen particles) pressed directly against them, even if they are in recessed locations. By contrast, the M cells in the tonsils and adenoids need to be able to sample, not just particles in food and water, but particles that are being breathed in, when there is nothing to press them against the M cells in the airways. Therefore, naso-pharyngeal M cells, and intestinal M cells, may have different arrangements and configurations, due to the very different conditions in which they function.

Returning to the main topic, the surface receptors which enable M cells to perform their sampling, monitoring, and particle-intake processes, are highly specialized, in ways which enable them to respond rapidly to particles carrying molecules that fall within a descriptive phrase, “pathogen-associated molecular patterns” (abbreviated as PAMPs).

The “PAMP” phrase can be better understood if the word “molecular” is omitted, so that the focus of the phrase is on “pathogen-associated patterns”. These include dozens of types of protein structures (the “dozens” number applies, if stringent criteria are applied; it would increase to hundreds, if less-stringent criteria are applied) which became “highly conserved” among numerous types of pathogens. Over the course of evolution, those “highly conserved” amino acid sequences – which usually are located in the most active and important “domains” of the proteins used by those pathogens – remained more constant than either: (i) amino acid sequences in other proteins, or (ii) other domains of the same proteins. The “highly conserved” domains of pathogen proteins were, in effect, sheltered and protected from the random mutations that constantly are being created by the process called “genetic drift, in other proteins and other protein domains. Some domains, in some microbial proteins, became “highly conserved” because they had reached, and settled into, a “sweet spot” (i.e., an optimally functional and efficient arrangement) for those domains, and could not get any better. Therefore, descendants of those types of pathogens were driven to preserve and sustain those particular domains, in those proteins, since random mutations would pull them out of the “sweet spot”, and would render the “awkward progeny pathogens” less efficient, and less able to compete successfully, against microbes having already-optimized highly-conserved domains in those proteins. As a result, any mutations that led to the emergence of new mutants, new strains, new species, and new genuses, in those families of pathogens, occurred mainly in *other* portions and domains of those microbial genomes, while leaving the “highly conserved” domains alone. A more extensive discussion of PAMPS is outside the scope of this application; if desired, more information (including

illustrations, lists, and examples of PAMPs among various types of microbes) can be found easily, via an internet search for "PAMP" combined with "pathogen".

Returning to the subject of M cells, when a "pathogen-associated pattern *receptor*" (i.e., one of the multiple types of pathogen-recognizing receptor proteins, located on the surface of an M cell) is contacted by a particle which has, on its surface, a corresponding "pathogen-associated pattern", the M cell receptor will bind to that protein on that particle, and the M cell will pull the particle inside, using a process called "phagocytosis", which is one type of a group of processes called "endocytosis".

As an important digression, the cellular process called "endocytosis" sits at the heart of how immune cells process both pathogens, and vaccine particles. It derives from the Greek roots for "internalizing something, by pulling or otherwise taking it into, a cell". Endocytosis is divided into two categories, called:

(i) pinocytosis, in which extremely small droplets of liquid are pulled in by a cell, very rapidly. This subcategory of endocytosis is not of interest, when discussing pathogens or vaccine particles, and it will not be mentioned again.

(ii) phagocytosis. "Phago-" is the Greek root for eat, and it implies an active or even aggressive form of eating, similar to the English words "devour" or "gobble". And, "cyto" is the Greek root for "cell". So, "phago-cytosis" is the process of endocytosis that occurs, when a cell pulls in a particle, such as a virus, a microbe, or a vaccine particle. Since phagocytosis is the only category of endocytosis that is of interest herein, the two words *phagocytosis* and *endocytosis* can be used interchangeably, in the settings and context that are relevant herein.

To qualify as phagocytosis, a cellular intake process must meet two criteria:

(1) The cell must expend energy, in order to bring the particle inside the cell. This effectively means that the cell is in control of the process, and is not being invaded by some mechanism being carried out by the particle or molecule.

(2) In addition, when the particle first enters the cell, it must be surrounded by a small segment of membrane which was created, at least in part, by taking some portion of the cell's outer membrane. Any small bubble of membrane material can be called a "vesicle", and when a vesicle is formed by the process of phagocytosis, it is called a "phagosome" (the term "endosome" also is correct, but it is less specific, less descriptive, and less helpful). That type of small enclosure helps protect cells against pathogens, since a phagosome will not contain (and will not give a newly-arrived microbe access to) any additional nutrients, which the pathogen might otherwise use, to begin reproducing, or doing its "dirty work" inside the cell.

In the very large majority of cases, a different type of small bubble-like enclosure, called a "lysosome" (which will be enclosed within its own bubble, made of the same type of membrane material) will merge with a phagosome, in a manner comparable to two drops of oil, floating on water, merging with each other to form a single larger droplet. The prefix "lys-" refers to breaking or splitting things apart; accordingly, lysosomes carry high levels of acidity, and aggressive digestive enzymes. The combination of high acidity, and "lytic" enzymes, will begin breaking apart any proteins they encounter, into small chunks that are no longer dangerous,

and into single amino acids, which the host cell can then use as nutrients for itself.

As mentioned above, when one of the "sampling receptors" on the surface of an M cell, in a MALT tissue patch, contacts a protein sequence on the surface of a particle which the M cell recognizes as a "pathogen-associated pattern", the M cell will pull the particle inside, using the process called "phagocytosis." That particle will be surrounded by, and fully enclosed within, a phagosome (i.e., a small bubble of membrane material, from the M cell's outer membrane). However, rather than waiting until a lysosome contacts and merges with this type of phagosome, an M cell will use specialized transport proteins, microtubules, and other internal cellular components, to rapidly push, pull, and transport any phagosome that has entered an M cell via a pathogen-recognizing receptor, through the M cell, toward the posterior membrane of the M cell (which also can be called the back side, distal, non-surface, or hidden membrane, or similar terms). That type of active and rapid transport, without any modification of the particle being transported, is called "trans-cytosis", which translates into "through (or across) the cell".

When the phagosome reaches the posterior wall of the M cell, the membrane which forms the phagosomal bubble will merge with the cell membrane, in a manner which ejects the particle (in an unchanged and "naked" form, no longer surrounded by a membrane bubble) into a "docking site" on the posterior side of the M cell.

In each M cell, the docking site on its basal/posterior/hidden side generally has the shape of a cup, or pocket, which is large enough to hold both an immune cell, and a pathogen, with enough room left over for the immune cell to take its time and perform its "sampling, analysis, and commitment" functions. Accordingly, dendritic cells (and, to a lesser extent, macrophage cells) travel to, and settle into, and then wait, in those M cell docking sites, in large numbers. They are waiting for an M cell to deliver a pathogen to them, so that they can then take over and process that pathogen, in a way which will lead to a suitable immune response.

Stated in other words, M cells function as delivery agents, and/or as "gated tunnels" (or "gatekeepers") which pass through the otherwise "tight junction" membranes that surround MALT patches. However, the "gatekeeper" term would be misleading, if it brings to mind a receptionist sitting quietly at a desk, outside an office, to prevent people from getting into that office unless they are invited. A better analogy would be a large, strong, physically imposing security guard, in a shopping mall, in a city that has been hit multiple times by terrorist bombings. If such a guard sees someone carrying a large backpack, and acting in a way that is not merely suspicious but alarming, the guard can grab that person, pull him inside a store, and say, "Come in here. I need to talk with you." Then, once he has the suspect inside the store, he can say, "Actually, it's not me that needs to talk with you. It's the guys on the other side of this door." And, with that, he shoves the suspect out through the back door of that storefront, into a security tunnel, where a set of military veterans and bomb disposal experts are waiting.

In a similar way, M cells actively grab particles which are carrying "pathogen associated patterns"; they rapidly pass those particles through the M cells; and, they eject those particles directly into the "waiting arms" of antigen-processing immune cells, which are waiting for exactly those types of deliveries, in the "docking sites" on the back sides of M cells.

When such a handoff occurs, a dendritic cell which receives a pathogen particle can begin acting, as soon as it encounters and grabs hold of the potentially pathogenic particle (which, in the case of the vaccines particles described herein, will be an entirely harmless particle, carrying a bag of valuables, but dressed up to look like a serious threat). At any *other* location in an animal body, when a dendritic cell detects and then grabs hold of a particle carrying one or more "pathogen associated patterns" which indicate that it is a potentially serious trouble-maker, the dendritic cell must shut down its other operations, and begin traveling toward a lymph node, so that it can locate, contact, and interact with B cells and helper T cells, which will take over and perform the next steps in creating a full immune response.

However, as mentioned above, one can define "lymph nodes" as "the specialized patches of tissue where T cells, B cells, and other immune cells gather together, so that they can work together, and cooperate, to create antibodies which will bind to the 'chunks' of foreign proteins that are brought to those lymph nodes by 'antigen-presenting' mobile immune cells." And, if that definition is used, then MALT patches are special types of lymph nodes, mounted on the surfaces of mucosal membranes. Therefore, if a pathogen is gripped by a dendritic cell which was waiting for that type of delivery, just below the surface, inside a MALT patch, that dendritic cell is already in a "surface-mounted" or "secondary" lymph node, and does not have to shift into a traveling mode, and go some distance to find a lymph node, which otherwise might take hours, or even days.

Accordingly, it is believed and assumed that a response by a dendritic cell which is already within a MALT patch (which already contains and include B cells and helper T cells) can, in many and perhaps most cases, be more rapid than a response that occurs outside a lymph node, in some other type of soft tissue; and, whenever fast-reproducing pathogens are involved, a faster immune response is a better response.

The traits and features described above make MALT patches ideally suited for responding rapidly, as a crucially important part of a "first line of defense" which evolved in mucosal membranes, to help defend animals against pathogens that enter animal bodies via air that is inhaled, or food and liquids that are swallowed.

It also should be noted that, in addition to having "functional" (or secondary, surface-mounted, etc.) lymph nodes on their exposed surfaces, mucosal membranes also have relatively large numbers of mobile immune cells (including macrophages, dendritic cells, B cells, and T cells) traveling in and through the shallow layers directly beneath all of the mucosal surfaces, because that is where much of the action occurs. As examples of the types of trouble that can occur in the shallow layers, which don't penetrate to deeper layers:

(i) small nicks and abrasions are often created inside the mouths of animals, by the act of chewing solid foods, including foods that often contain rough fiber, bark and wood-like chunks, thorns, bones, skins that are thick enough and tough enough to protect the food against pests and predators, etc;

(ii) nicks, abrasions, and small lesions also are frequently created inside the nasal cavities, when particulates (such as sand and dust) and/or acidic, corrosive, or other troublesome

particles (such as soot) are inhaled; and,

(iii) numerous types of pathogens have evolved with ways to actively grab hold of, and then penetrate into, the epithelial cells that cover the surfaces of mucous membranes.

Therefore, even though most of the focus of the "mucosal vaccine" discussion herein will focus on MALT tissues (and especially NALT tissues, since MALT patches inside the mouth and nasal cavity are the most readily and easily accessible subcategory of MALT patches), it should be recognized that the same types of vaccine particles described below are likely to also be transported into "sub-epithelial" tissues, in the mouth and nasal cavity.

However, there is a crucial distinction between:

(i) what happens to invasive microbes (and vaccine particles) that are "grabbed" and processed by dendritic cells that are waiting for deliveries, on the "back side" (also called the "basal" side or surface) of an M cell; versus,

(ii) what happens to invasive microbes (and vaccine particles) that are "grabbed" and processed by dendritic cells (or macrophages, or B cells, which are the other main types of mobile immune cells) that are in "subepithelial" tissues that are NOT in MALT patches.

As described in more detail below, if an invasive microbe or vaccine particle is "grabbed" and processed by a dendritic cell that was in a MALT patch, waiting for a delivery from an M cell, the process triggered by that event usually leads to formation of a "secreted IgA antibody dimer", which is a highly specialized type of antibody, as described below (and, to help establish the importance of that fact, *vaccine particles which can effectively trigger and drive the formation of secreted IgA antibody dimers* are the primary focus of this invention, as described below). By contrast, if an invasive microbe or vaccine particle is "grabbed" and processed by a dendritic cell that was NOT in a MALT patch, and which instead were merely "nearby", in a much more generalized "sub-epithelial" tissue layer, then that process will probably lead to the formation of standard, classic, Y-shaped IgG internal antibodies; and, there are lots of ways to create those, using lots of different types of vaccines that have been known for decades.

Accordingly, the value and the focus of the invention herein, is that it enables the creation and use of vaccine particles which will trigger and drive the formation of secreted IgA antibody dimers, in a way which has never previously been available, rather than merely offering yet another way to create standard IgG antibodies, as can be accomplished by numerous other types of vaccines that have been known for years. The fact (as described below) that these new types of vaccine particles can also trigger the creation of standard IgG antibodies – *in addition to* secreted IgA antibody dimers – should be regarded as a bonus, since it will enable a single vaccine inoculation, performed topically, with no needles or other invasive methods, to create a balanced, bi-functional, "two-handed" immune response, as described below.

The structural and functional differences, between standard Y-shaped internal IgG antibodies, versus secreted IgA antibody dimers that float and act in mucosal secretions, outside of any cells and tissues, must be understood, to grasp the nature and importance of this current invention. Accordingly, conventional internal Y-shaped antibodies (and how they actually function, which is different from what most people assume and believe) will be described in the

next section; then, that will be followed by a description of how (and why) secreted IgA antibody dimers have both: (i) a very different structure, and (ii) a very different function.

HOW IMMUNE CELLS MAKE Y-SHAPED IgG ANTIBODIES

As briefly discussed above, the main function of a classic Y-shaped IgG antibody, in blood or soft tissue, is to grab hold of something that looks foreign, and then change the shape of its Fc "stem" portion, in a way which (with additional help from complement proteins, as also mentioned above) send out signals to recruit any nearby mobile immune cells to come over and help. Accordingly, Y-shaped IgG antibodies can be described as having a "tag and flag" function (while secreted IgA antibody dimers – having two stems wrapped together, with four "active arms" at the ends of each dimer – have a very different "grab and drag" function, as discussed in more detail below).

The two "arms", at the top of a Y-shaped IgG antibody, are called the "variable" fractions, abbreviated as the Fv portions. They are "custom sequenced" by B cells, so that any particular antibody will bind, selectively, to only a very few protein sequences (which normally are antigenic epitopes, on pathogens). The ability to make literally billions of different antibodies, by shuffling segments of DNA around within a carefully-limited "hyper-variable" region of a specific chromosome, is unique to B cells; and, the process which B cells must go through – to figure out how to make antibodies which will bind tightly and selectively to some new pathogen that no B cell has ever previously encountered – is highly complex. It took decades for scientists to figure it out, partly because it involves several different cell types, and several major cycles (or iterations), where each successive cycle can be likened to an elaborate dance, where the losing suitors must die. And, except in rare and fortuitous occasions, a strong presumption arises that the only B cells which can make new types of antibodies, against newly-encountered invading pathogens (or vaccine particles), are those which are dwelling, at the time, in a lymph node, rather than patrolling through soft tissues, because lymph nodes are the only locations where: (i) enough helper T cells will be close enough, and concentrated enough, to be able to help; and, (ii) enough B cells are present, with all of them trying to make the best candidate antibodies, to allow the process to work adequately.

When a macrophage or dendritic cell shows up at a lymph node, carrying a chunk of foreign protein (usually between 8 to 12 amino acids long, and presumably from a pathogen, but possibly from a vaccine particle) which is mounted on a special "presentation plaque" (called an MHC-2 protein), a set (or cluster, series, etc.) of B cells will migrate to the "antigen presenting cell". The B cells will press up against the chunk of MHC-mounted protein which is being carried by the "antigen presenting cell" (presumably, only one B cell at a time can do this, for any specific chunk of protein, but it is not known how many different chunks of foreign protein can be carried by any "antigen presenting cell"). Specialized types of proteins on the surfaces of the B cells are involved, and those which have the highest level of "binding affinity" for the chunk of newly-presented foreign protein, will send a signal to a complex DNA-rearranging set of proteins inside the B cell, which are responsible for handling the "hyper-variable"

chromosome region of that particular B cell.

In each B cell that becomes involved in that process, inside a single lymph node, those proteins, and the hyper-variable segment DNA, will work together, to create a first antibody candidate. As mentioned above, it will not be a complete antibody; instead, B cells can create what are, in effect, smaller "trial size" antibodies (which were given the name "IgM" antibodies), which can be made more rapidly and easily than full-size IgG antibodies. The B cells which create those IgM trial-size antibodies will "offer" those new IgM antibodies to certain types of T cells, which also have somehow encountered and responded to the antigenic protein sequence which was brought to the lymph node by a macrophage or dendritic cell.

Those B cells which have formed the most promising IgM candidates, which can bind with a respectable level of strength and selectivity to the antigen sequence, are stimulated by signals from one or more T cells. Those signals use molecules called "cytokines", which are similar to hormones, but specialized for the immune system, as described in more detail below.

The B cells which receive stimulating cytokines, from the "supervising" T cells, will live, and keep working; and, B cells which do not get any of those approving and encouraging cytokines will shrivel and die, using a process of "programmed cell death" called "apoptosis", which gets them out of the way. In that manner, the "winning B cells" that were selected by the first cycle, will become the contenders, in a second cycle of competition and selection. They will begin forming a "second round" of IgM antibody candidates, which will be variants of the first candidates, and the "second round IgM contenders" will then go through another process of evaluation and selection, involving the T cells.

After several cycles of that type of selection – with each cycle usually leading to better-binding antibody candidates – a T cell which detects a potently-binding IgM antibody, on a particular B cell, will shift into a "final approval" signaling mode. The B cell(s) which are activated by those signals will begin reproducing, to make more copies of the selected and activated B cells. Those new copies of the "winning" B cell will leave the lymph node, travel in whatever direction certain signaling cytokines tell them to travel, and begin making full-sized and complete IgG antibodies, instead of the smaller "trial-sized" IgM antibodies.

As mentioned above, that process is extremely complex, and it literally requires certain types of cells to "rearrange their innards" for a chance to survive and reproduce. Videos which depict and explain those processes include youtube.com/watch?v=GXVLdbkRkhw (Dr. John Looney, T Cell Activation and Control), and youtube.com/watch?v=TMnkihN6zVM (Armando Hasudungan, MHC II Processing).

HOW MALT PATCHES MAKE AND SECRETE IgA ANTIBODY DIMERS

The same process that is used to make classic Y-shaped IgG antibodies, inside "conventional" internal lymph nodes – i.e., requiring B cells and T cells to receive a "chunk" of a foreign protein from a mobile "antigen-presenting" cell, and then going through two or more cycles of trying to make good antibodies which will bind to that foreign protein, by selecting the B cells that make the best candidates, and giving special cytokines to those selected B cells,

which will get them to keep trying to make better candidates, until a good one is finally recognized and selected – also is used to make each Y-shaped IgA antibody component, in a MALT patch. That is only part of the process, and it needs to keep going, through additional steps; however, it does not need to be repeated again from the very start, since the same B cells began making the Y-shaped IgA monomers will continue making more of them.

As described and illustrated in Woof and Russell 2011 and Wei 2021, IgA antibodies are initially created inside B cells, in the same Y-shaped monomer form as IgG antibodies. However, unlike IgG antibodies, the Fc portion of an IgA monomer has a "tailpiece" at its "bottom" tip, with an exposed cysteine residue. With help from a chaperone protein called MZB1, two IgA monomers will bind (via disulfide bonds) to a third molecule called the J chain, which has two exposed cysteine residues on opposite surfaces. That forms an "initial" dimer, which is secreted by the B cell. The J chain component will then be recognized by a surface receptor, called the pIgR receptor, on the basal surface of an epithelial cell, and the dimer will be pulled inside a cell, by that receptor. A portion of that pIgR receptor will wrap around the J chain and the stem components of the initial dimer, to create a "secretory component" which strengthens and stabilizes the antibody dimer, and imparts additional benefits to it. The stabilized dimer (including a J chain, secretory component, and two IgA antibody monomers) will then be detached from or secreted by the epithelial cell, and released into saliva or other mucosal fluid.

It is presumed and believed that most (and perhaps nearly all) B cells which create and secrete "loosely joined" IgA dimers – connected by J chain peptides, but not yet having a pIgR "secretory component" wrapped around the coupled stem components – are either residing in MALT patches, or have "gravitated" or "homed" to the shallow fluid layers beneath MALT patches, or possibly passed through MALT patches and received some type of signal which instructed them to make IgA monomer precursors, instead of (or possibly in addition to) making IgG monomers. This factor helps emphasize and highlight the crucial and central importance of MALT patches, in creating secreted IgA antibody dimers. However, B cells are mobile, and can leave a patch of MALT tissue, and can continue creating and secreting IgA monomers, and "initial" IgA dimers, even after a specific B cell has left a MALT patch.

B cells which make IgA monomers also secrete substantial numbers of those monomers into blood and lymph; in vernacular terms, it is, to some extent, merely a matter of chance, and probability, as to whether any specific IgA monomer will happen to encounter an MZB1 chaperone protein first, which will then introduce and attach it to a J-chain protein, or whether it will encounter a cellular outlet first, and leave the B cell while still in monomer form. When released, IgA monomers function in the same way as IgG antibodies, by "shape shifting" their Fc stem components into active signaling mode (leading to complement protein fixation, and immune cell recruiting), when they become affinity-bound to a particle or protein.

While the presence of IgA monomers can make it more difficult for researchers to analyze IgA dimer concentrations, the output numbers and concentrations of IgA monomers, versus IgA dimers, is not highly important. Instead, if IgA antibodies which will bind specifically to an antigen sequence carried by vaccine particles (as can be tested and measured,

by the types of tests described below) are being created and released by B cells, then that vaccination was successful, regardless of whether the IgA antibodies are in either monomeric or dimeric form, and attention can and should shift to the question of whether those vaccine particles will indeed help protect inoculated animals, against the pathogen which the antigens were derived from.

In addition, as discussed below, a presumption will arise that, if vaccine particles having MALT-targeting peptides on their surfaces are inoculated into animals via a topical mucosal route, and if they are able to trigger the formation of either IgG or IgA antibodies which will bind to the antigen sequences on those vaccine particles, then that inoculation was successful, and it can be left up to the B cells to somehow determine how many IgG antibodies they will form, versus how many IgA antibodies they will form, in response to that inoculation. Once that level of achievement has been reached, for any candidate vaccine which is carrying MALT-targeting sequences and which is applied topically to one or more mucosal surfaces, important questions will shift over to things like: (i) How much protection can that vaccine provide, against the pathogen its antigen was derived from?; and, (ii) What dosages should be administered to the animals or people that will be receiving that vaccine, to maximize its efficacy and protective benefits?

Anyone who wishes to know more about how IgA antibodies are converted into elongated dimers can find full color drawings which offer a good introduction, in the Wikipedia entries for “J chain” and “secretory component”. A more detailed description, also with illustrations, is contained in Brandtzaeg 2009, which is a review article entitled, “Mucosal Immunity: Induction, Dissemination, and Effector Functions.” A full copy of that article, made available in pdf form as a public service to researchers in this field, is available at no charge from onlinelibrary.wiley.com.

PRIOR ATTEMPTS TO DEVELOP MUCOSAL VACCINES

As used herein, the phrase *mucosal vaccines* refers to vaccines that are designed and formulated to be directly applied to some type of mucosal membrane surface, in a “topical” manner. As used herein, “topical” inoculation (or application, administration, infusion, or any other suitable term, depending on the specific method employed) refers to any method of administration which causes the vaccine particles to directly (and, gently, with no particular ‘driving force’ that will cause pain or irritation) contact the outer surface of a mucous membrane.

Although there may be some gray zones, or boundary zones, which cannot be clearly and directly classified into either “topical” or “injected” categories, the definition of “topical” offered above is intended to avoid overlapping with vaccines that are injected. As used herein, injection refers to any method that uses some type of device to forcibly and mechanically drive vaccine particles through an “outer surface” (which, in most cases, is likely to be an epidermal (dry skin) surface), into the soft tissues below that outer layer. Most conventional *injection* devices use needles or high-pressure jets; however, the definition of “injection” is broad enough to also cover and include, for example, devices containing multiple tiny micro-needles, which are designed to

feel like sandpaper, but which actually penetrate the outer/surface layer, and push vaccine particles down into the underlying layer(s) of soft tissue.

Topical administration of mucosal vaccines can be accomplished by variety of methods, depending on which particular category, set, and location of mucosal membranes are being "targeted" by the mucosal vaccines. It can be done via nasal spray, if the vaccine is intended to protect against a pathogen which infects the nasal cavity and/or respiratory tract (such as influenza, corona viruses, pneumonia, tuberculosis, etc.). Alternately, a liquid formulation comparable to a mouthwash can be gargled, sucked, or dissolved in the mouth, so that the particles contained in that liquid or device will directly contact the tonsils and several other NALT tissue patches, which collectively are known as "Waldeyer's ring".

If a vaccine is intended to protect against a pathogen which infects the digestive tract (such as cholera, dysentery, or a polio or rotavirus), the vaccine can be put inside a capsule which will not be degraded by stomach acid, but which will digested and dissolved, by digestive enzymes, after it reaches the intestines. Keratin, the structural protein in hair and fingernails, formerly was used for that purpose, but cheaper polymers – which can be manufactured in bulk, rather than having to extract them from biological sources – are now available, and generally preferred. Alternately, an enema or suppository can be used, to enable vaccine particles to "topically" contact and interact with intestinal surfaces, and "Peyer's patches".

In addition, mucosal vaccines are administered to non-human animals by various other methods that are not widely known to people outside that specialized field. As just two examples, mucosal vaccines are sometimes added to salt blocks and various types of "bait foods" that will be licked or eaten by wild or herded animals; and, they are sometimes put into mists, via "atomizers", in enclosed chambers (such as for poultry).

All of those modes of topical administration, to mucosal surfaces, are well-known, and still more options are described below, under the "Detailed Description" of this invention.

On a theoretical level, there are numerous reasons why mucosal vaccines: (i) should be able to provide various advantages over vaccines that require injection using needles; and, (ii) should be able to complement and add additional options to the various types of injectable vaccines in use today. As a brief recap, those reasons include:

(a) Mucosal surfaces cover larger areas than dry skin, and they are active and fertile targets for pathogens; as a result, nearly all pathogens (excluding blood-borne) have evolved in ways that enable them to attack and invade mucosal membranes;

(b) Animal bodies have evolved with secreted mucosal IgA antibody dimers which are very different from conventional internal IgG antibodies, in both shape and function;

(c) Vaccines which are injected almost never lead to mucosal antibody formation, even though mucosal antibodies could, if created in response to a mucosal vaccine, be highly useful in fighting off infections, especially by pathogens which infect upper respiratory membranes and/or the lungs; and,

(d) many types of viruses and bacteria can reproduce very rapidly (i.e., in less time than it takes for a surface epithelial cell to create "I need help" signaling molecules, and then move

them to its basal surface, and then wait for an immune cell to detect and respond to those signaling molecules). Therefore, those types of pathogen can often reproduce hundreds or thousands of copies of themselves, without ever encountering or having to deal with any IgG antibodies.

However, despite their potential advantages, mucosal vaccines have been sadly limited, and severely lacking. Despite numerous efforts to create mucosal vaccines, only a very few mucosal vaccines have ever been developed and commercialized successfully. A fairly recent comprehensive listing, in a 2012 review (Rhee 2012, at page 51), took less than a single paragraph, and didn't even merit a table, to list them. In additional to the small number that have been approved for sale and use, various comments below summarize or cite articles published in scientific or medical journals, describing efforts to create mucosal vaccines which are still in the "early research" stages, and may never be commercialized.

Accordingly, there is a general belief, expressed in private among immunologists, that the only truly successful and effective mucosal vaccine ever created is the polio vaccine. However, even polio vaccines cannot be regarded as a true success, unless someone knows the story behind them, and recognizes that two entirely different types of oral polio vaccines must *both* be given to any person, to provide truly effective protection. Both types of vaccines were initially developed in the 1950s (although Sabin's work continued well into the 1960s), with the competing (and bitterly competitive, and rivaling) lead scientists being Jonas Salk, who used chemically-killed mixtures of the three major strains of dangerous polio viruses, and Albert Sabin, who used "live-but-attenuated" polio viruses.

The Sabin vaccine is more effective, and offers longer-lasting protection, since it contains "live" viruses (some experts prefer the term "fully viable", since viruses are not actually "alive" as that term is normally used). Unlike chemically-killed viruses, "live viruses" can and will begin to spread around, into various organs and tissue types, and the immune system is better at defending against "spreading infections", than defending against things that are already clearly "dead" (or "non-viable").

However, the "live viruses" in the Sabin vaccine pose serious and genuine risks that one or more of the viral descendants, which reproduce from the "live" viruses in a Sabin dosage, might revert to truly pathogenic form, which can inflict a life-long crippling or hobbling infection on the person who was given those viruses, in a vaccine. During the height of Sabin vaccine usage, in the mid-1960s, a dozen or so people became infected with genuinely severe and crippling cases of polio, each and every year, just in the United States, soon after being inoculated by the Sabin vaccine, with its still-viable (and potentially dangerous) viruses.

Therefore, the regimen that came to be endorsed and recommended required giving someone the Salk vaccine first, and then waiting a month or so, for that person to develop a level of protection which could protect against "Sabin mutants". Then, that same person could be given the Sabin vaccine, with very little risk, since that would provide better, more long-lasting protection against polio infections, than the Salk vaccine.

And, so . . . the only oral vaccine that is regarded by specialists as being "truly effective"

needs to be heavily conditioned, and "asterisked", by a warning that it is indeed effective, but only if two entirely different vaccines against it are administered, with the Salk version being given first, and the Sabin version being given also (but only after waiting until the Salk version has had enough time to take full effect). Most vaccine experts don't voluntarily choose to discuss that unpleasant complication; instead, they prefer to simply say that the polio vaccine is the best and most successful mucosal vaccine that has ever been created, and that statement is entirely true. And, indeed, shifting combinations of the Salk and Sabin vaccines did indeed nearly eradicate polio, as a disease . . . at least, in the United States . . . at least, until it suddenly reappeared again, as a truly dangerous threat, in upstate New York, in 2022. And so, any comments that polio vaccines were and are "the best mucosal vaccine ever created" need to have qualifications and caveats added to them, to render them truly accurate and useful.

Furthermore, most mucosal vaccines cannot be administered to the very young, the elderly, or immuno-compromised patients, even though those three classes of patients are often likely to need, most acutely, whatever protection mucosal vaccines might be able to provide. This unfortunate limitation arises from the fact that, in the past, most mucosal vaccines had to contain "viable but attenuated" pathogens (i.e., living pathogens which have been weakened, but which are nevertheless alive, viable, and able to reproduce), in order to be even somewhat effective.

In addition, as discussed in more detail in the section on adjuvants, below, very little work is being done to try to translate research on mucosal vaccines in animals, to the development and use of mucosal vaccines in humans. This factor arises largely because most of the "adjuvants" (described in more detail below) which are included in mucosal vaccine formulations tested on animals, are so aggressive and toxic (in order to generate useful statistics, despite limited populations and high cost-per-animal testing expenses) that they cannot be used in humans. As examples, the most widely used adjuvants, in tests on livestock and other animals larger than rabbits, are cholera toxin, and a "heat-labile" toxin from pathogenic strains of *E. coli*.

Accordingly, there is not a lot of research being done into vaccines that can be administered directly and topically, to mucosal membranes. To help illustrate that statement, US patent 6117632 (Mahoney; filed in 1996, and issued in 2000) described how to use phage display libraries in specialized screening tests, to identify and isolate those particular phage particles which are actively transported into "Peyer's patches" in the intestines (as mentioned above, "Peyer's patch" is the name that was given to MALT patches in the intestines). However, it appears that no serious and sustained effort was ever made, by any vaccine company, to expand that proposal into any actual mucosal vaccines that might be able to help the public. That patent expired years ago, in 2016, without ever accomplishing anything useful.

One of the few mucosal vaccines that has been developed and used with even a modest degree of success is an influenza vaccine sold under the trademark FLUMIST(TM) by MedImmune Vaccines, Inc. (a subsidiary of AstraZeneca). It is described in articles such as McCarthy 2004, and on websites such as www.flumist.com, as well as fda.gov and rxlist.com (both of which have search boxes that allow anyone to search for items containing "Flumist").

The FLUMIST vaccine contains “live attenuated vaccine particles”. Those particles are living influenza viruses, which can reproduce and multiply, in susceptible cells, in ways which can “amplify” the effects of a one-time dose of the vaccine.

The fact that certain types of specially-selected live intact influenza viruses can be used safely, as mucosally-administered vaccines, arises from an unusual factor. The viral strains that were used to create FLUMIST vaccines descended from strains which had been discovered to infect only certain types of cells, which usually are described as “temperature-restricted” or “cold-adapted” cells. These types of cells occur mainly on the surfaces of the membranes in the nasal cavity, where their temperatures normally (and especially during daylight hours, in winter) are substantially lower than interior body temperatures, because the outer surface tissues, in the mouth, nasal cavity, and bronchial regions, are frequently and repeatedly chilled by the simple act of breathing in cold air (including *very* cold air, in near-arctic regions). When scientists began studying why influenza was heavily dependent on seasons, “temperature dependence” was an obvious candidate as a contributing factor, and it was high on the list, as studies were being planned and organized. During those studies, it became clear that the levels of temperature dependence varied widely, among different strains of influenza viruses; and, since the scientists wanted better tools to help them study those factors, they began to isolate strains of influenza viruses which were highly susceptible to temperatures.

That research, and the identification of a specific viral strain which was strongly limited by the temperature of the cells it could infect, led to the development of spray-administered FLUMIST vaccines. The “host” strain, which is engineered to carry a new set of antigens (which must be identified and updated before the beginning of each flu season, in each of the northern and southern hemispheres), cannot infect the cells in the lungs, or in the layers beneath the outermost membranes in the nasal cavity, because those cells are outside the temperature range in which the host strain can reproduce. Therefore, that strain cannot cause the types of severe and widely-dispersed discomfort and other symptoms that are caused by normal influenza infections.

As with all other influenza vaccines, the FLUMIST vaccine is only partially effective; efficacy estimates usually range from about 40% to about 70%, in various years (as measured by how many people become infected by influenza, *despite being vaccinated*). And, a new and updated version of the FLUMIST vaccine should be administered each and every year, at or near the beginning of the flu season (i.e., the onset of cold weather) in each country, since prior vaccines are not likely to provide sufficient protection against those new mutants and variants which will pose the greatest threats of major outbreaks during that season (indeed, the criterion of “greatest threat” points directly toward mutants which are able to avoid antibodies that were generated in response, not just to prior vaccinations, but to prior infections as well).

Furthermore, the FLUMIST vaccine cannot (lawfully) be used on certain classes of patients, such as the very young, the elderly, and anyone who is immuno-compromised; and, THOSE are the groups and types of patients who are at the greatest risk of either or both of: (i) becoming infected by influenza; and/or, (ii) suffering severe problems, up to and including permanent impairment, brain damage, or even death, if they do become infected by influenza.

The only noteworthy mucosal vaccine known to the Inventor, for humans, which has been created after about 2000, is an orally-ingested vaccine against rotaviruses, a class of viruses which create major digestive problems and diarrhea among infants, especially in tropical regions. However, the first attempt to release and use that vaccine led to major problems, and that vaccine had to be withdrawn from the marketplace, as described in articles such as Murphy 2003, after it was realized that it was causing a severe and potentially fatal intestinal problem called "intussusception" – which must be corrected surgically, as quickly as possible – in significant numbers of the infants who received that vaccine. When the sponsoring company re-designed and re-tooled that effort (in a vaccine that is now being sold under the trademark ROTA-TEQ), the company – rather than adding any transport-enhancing proteins to the surfaces of those vaccine particles, as suggested by the Mahoney '632 patent, mentioned above – reduced the number of rotavirus antigen proteins that the virus particles carried, and based their decisions on hopes and assumptions that those antigenic proteins – even though reduced in number – would still be able to help promote uptake of the vaccine particles, by Peyer's patch cells in the intestines of vaccinated infants.

As a final comment, in this brief survey of mucosal vaccines, the history of how vaccines were created against polio viruses, offers a classic set of lessons and examples, to anyone who wants to better understand the halting and unsteady yet cumulative progress of science, and the challenges of proving that a new vaccine will be sufficiently "safe and effective" to merit public use. Among other things, it provides an illustration of the contest between "killed pathogen" versus "live attenuated" vaccines, in the form of angry and bitter battles between competing teams working with "the Salk vaccine" versus "the Sabin vaccine". It is described in detail in *Polio: An American Story* (David Oshinsky, Oxford Univ. Press, 2005), which won a Pulitzer Prize for the best non-fiction book of that year.

The story of Jonas Salk also offers a useful warning, to scientists who are inclined to take as much credit as they can for themselves, without sharing it with those who helped and enabled them. Salk's story is every bit as interesting as Robert Oppenheimer's, as portrayed in the movie *Oppenheimer* (which, for reasons unknown, used a colleague named Strauss as "the bad guy", while ignoring the much more important role of Edward Teller); so, anyone interested in science or medicine might hope that Salk's story also will become the subject of either a movie, or at least a TV mini-series. Salk was lionized by the public, but he died an angry, frustrated, and bitter man, wondering whether he would have been more respected – by his peers – if he had treated his underlings and co-workers with more respect and generosity, and if he had shown better control over his need to try to grab and claim all the publicity, and credit, for himself.

Two facts can help illustrate what he did, and how it ended up affecting him. The first fact was this: even worse than being passed over, every year, for a Nobel Prize, was the hard and unhappy declaration that he also was passed over, every year, for membership in the National Academy of Sciences (even though they can admit more than 100 new members, every year), and the Institute of Medicine (now called the National Academy of Medicine, which can admit 80 new members, ever year). Those organizations encourage and promote cooperation,

communication, and collegiality, and Salk knew, all too well, that he had been painted as the stingy and selfish *opposite* of those things, ever since he had refused to even acknowledge the presence – let alone the contributions – of the co-workers who had been working super-hard for years to help support his research, when he – and only he – announced the results of a major multi-national trial, which proved that the polio vaccine could indeed work.

The second fact was this: after he had managed to help create and find funding for a gleaming monument to himself (named *The Salk Institute*, of course; he would not have had it any other way, and pictures of it are easy to find, with beautiful and very impressive buildings on a beautiful coastal bluff, north of San Diego), he was pushed aside, as an active manager, and was forbidden to have anything but a fund-raising and ceremonial role in it, within less than 5 years after the buildings were finished. Surely some screenwriter can make something which would be both entertaining, and educational (in a good way), out of that story line; indeed, a scene showing a lonely and embittered man, walking down the middle, between those two rows of buildings, knowing he helped build them but is no longer welcome in either of the two rows of buildings he helped build, could be a marvelous bit of cinematic staging.

In addition, anyone who studies the history of polio vaccines will learn about a massive and terrible failure, involving contaminated and infective "Salk vaccine" preparations sold by a company called Cutter, which aggressively infected multiple thousands of recipients with severe cases of polio. Due to the massive public and press attention to that failure, the efforts to handle the crisis, the work and planning that was done to prevent it from ever happening again, and the fact that it forced the government and private enterprise into a form of genuine partnership with each other, "the Cutter fiasco" can be said to rank alongside the development of penicillin, and X-rays, as the three events which, together, created "the start of modern medicine."

Numerous attempts to develop other types of mucosal vaccines have been tried, and have failed. This includes, as just one example, efforts by a company called Oravax Inc. to develop several types of mucosal vaccines. That company effectively collapsed in 1998, and was acquired by Peptide Therapeutics Group PLC, which later was taken over by Acambis PLC (www.acambis.com), which apparently is no longer attempting to develop any mucosal vaccines.

More information on the history of mucosal vaccines is available in review articles such as Vajdy 2004, O'Hagan 2004, and Holmgren 2005. Reviews that focus on animal usage include Meeusen 2004; reviews that focus on efforts to develop mucosal HIV/AIDS vaccines include Belyakov 2004 and Stevceva 2004. Specific challenges that must be overcome by nasal vaccines, and attempts that have been made to overcome those challenges, are discussed in articles such as Eriksson 2002, Kiyono 2004, and Mestecky 2005.

In summary, the current state of mucosal vaccines, prior to the invention described herein, can be described as, "Not nearly as good as it should be, and needs to become, to help people fight off things like yearly influenza epidemics, the still-ongoing corona-virus problems, and the 'tripledemic' (COVID, plus influenza, plus respiratory syncytial viruses) that filled hospitals across the U.S. to near-capacity, during the winter of 2022-2023."

An additional cluster of prior art involves efforts to make mucosal vaccines, using a

specialized class of viruses called “phages” as the starting material. That art is described and discussed below, following the next sections, which explain what phages are, how they have been studied, and how they have been used.

Attention will now shift to a class of harmless, non-pathogenic viruses which became extremely useful research tools, and which are believed to offer the best types of “core particles” which can be modified, to turn them into the vaccine particles of this invention.

PHAGES (aka BACTERIOPHAGES): A BRIEF HISTORY

Since one approach to making the vaccine particles of interest herein uses specialized viruses called bacteriophages (today, they usually are just called phages), some background information on phages is provided herein. Anyone already familiar with phages, and the history of how they were discovered, can skip this section.

Before the 1930’s, when electron microscopes were invented and finally enabled researchers to see and study viruses, scientists used the term “virus” to refer to any invisibly small agent which appeared to have the traits of being both “alive,” and dangerously infective. The terms “virus” and “virulent” both arose from the same Latin root as “venom”, and initially referred to anything poisonous. As medical knowledge slowly progressed through the Middle Ages, “virulent” came to refer to any poisonous or dangerous substance from a diseased person (usually in a liquid or pus-like form), or from the site of an unhealed injury, or from some other foul-smelling or other noxious source, which contained something that could create a disease in previously healthy people or animals. Later, after microscopes enabled humans to see bacterial cells (beginning in the 1600’s), scientists gradually realized that some types of diseases (such as smallpox, polio, and herpes sores) were spread by microbes which could not be seen, even with the best microscopes, because they were much smaller than bacterial cells. Accordingly, the word “virus” became a catch-all term, to refer to dangerous and infective “whatevers” that were so small that they could not be seen, even with the best light microscopes of those times.

By the 1820’s, craftsmen who were skilled in working with clay and ceramics had developed unglazed ceramics, comparable to modern clay flower pots, which would allow water to pass through, but which would not allow passage of the microscopic “animalcules” which could be seen under light microscopes. That step forward was soon recognized as a major advance, since it allowed filtered and relatively safe drinking water to be obtained, even from sources that were known to be dangerously contaminated. Those types of ceramic filters are still used today, in many rural parts of the world (and by some backpackers, soldiers, etc.) to obtain drinkable water.

However, because water from some locations could still cause serious illnesses, even after it had been passed through one of those clay filters, scientists gradually realized, during the 1800’s, that: (i) some types of bacteria were dangerous mainly because they secrete toxic molecules which, unfortunately, could pass through the ceramic filters; and, (ii) some known diseases – such as rabies, herpes, and polio – could be caused by the agents which were called viruses, because they were clearly “virulent” and could reproduce, even though they were too

small to be seen.

In 1896, British researcher Ernest Hankin published the first report of an apparently benevolent and useful virus. He had found it in water taken from the Ganges River in India, a notoriously polluted river which caused cholera among nearly anyone who swam in it who was not from India. Hankin – a skilled researcher who had trained with both Louis Pasteur in France, and Robert Koch in Germany – reported that it had the traits of a virus, and could kill cholera bacteria.

Although that report spurred some minor interest in the subject, it was not converted into anything useful until 20 years later, when World War I triggered a desperate search for improved antibiotics. That search led to a breakthrough by Felix d'Herelle, who ended up selling millions of doses (in liquid form, which was swallowed) of a dysentery-fighting bacteriophage to the French army. Although no one knew what kinds of microbes were in those doses, they knew those microbes could protect soldiers against dysentery, and yet, were harmless to animals or humans (and, it should not surprise anyone that counterfeiters and frauds quickly sprang up, who also sold slightly cloudy and murky water to the armies, which could not protect those armies against anything).

The ability of "living but invisible" agents to potently kill certain types of dangerous bacteria could be proven, clearly and repeatably, by either of two types of demonstrations. In one type, a few very small drops of liquid containing the invisible agents were added to a jar containing water with a high concentration of a known pathogen (such as the bacteria which caused cholera, dysentery, or some other known disease); the jar was allowed to incubate for a day or two; and, it could then be proven that the pathogenic bacteria in the jar were no longer viable or infective, and had been killed, by something which had to be reproducing, in order for such a small initial quantity to kill so many bacterial cells – not just in the first jar, but in second, third, and subsequent jars, when small samples were taken from the prior jars.

In the second type of demonstration, drops of liquid containing the invisible agents were spread, at very low density, across "lawns" of bacteria which were growing on nutrient gels in shallow dishes. By the following day, the invisible agents would create circular colonies, which were visible as clear spots scattered across the bacterial lawns growing on the remainder of the gel surface. Since those clear visible circles would continue to grow, during the following days, it was apparent that they contained some sort of microbe that was reproducing, using the bacteria as its source of food.

After those findings were confirmed, scientists chose the term "bacteriophages" to describe those unknown microbes. The root word "phage" is the Greek word for "eat", and it implies an active and aggressive form of eating, comparable to "devour." So, "bacteriophages" referred to unknown microbes which could "devour" bacteria.

Those studies were not driven by mere curiosity. Penicillin was not developed into a practical antibiotic until the mid-1940s, when World War Two triggered another surge of desperate searches for better antibiotics. So, until the late years of World War Two, the best "antibiotics" were mostly dilute versions of outright poisons, such as arsenic, cyanide, mercury,

or severely unpleasant sulfur compounds (which gradually were improved, to create the first "sulfa" drugs). Therefore, "bacteriophage therapy" became a large and important branch of medicine during the 1920s and 1930s, and even throughout World War 2 (while penicillin was still being studied and developed, and then, while the meager early supplies were too small to meet the huge needs for it). If a patient was suffering from a serious bacterial infection which nothing else could cure, doctors or researchers often directly administered, to an infected site, whatever types of "bacteriophages" they believed might best be able to kill the types of bacteria which were causing that infection. Alternately, if a patient was suffering from a disease which attacked the digestive tract, such as cholera or dysentery, physicians would have the patient drink a solution containing phages which might be able to kill the bacteria causing that disease.

That practice has also been used successfully, recently. An account of one such episode, in 2016, can be found by an internet search for "Strathdee" combined with "Acinetobacter". Steffanie Strathdee is an epidemiologist, specializing in infectious disease, and her husband was dying from an antibiotic-resistant strain of a pathogen in the genus *Acinetobacter* (he became infected while in Egypt). She knew who to contact, because of her professional work, and nothing else was helping, so she persuaded the FDA to go along with her efforts, and began contacting phage experts. They tested their collections, to see which ones could fight off that particular pathogen, and they found some promising strains, which were infused into her husband, and which led to his recovery.

After the development of electron microscopes (in the 1930s) enabled researchers to see that "bacteriophages" were a specialized class of viruses that could infect only certain types of bacteria, they dropped the prefix "bacterio-", and the common names for that class of viruses became simply "phages".

INOVIRUS AND FILAMENTOUS PHAGES

By roughly the mid-1970's, as research into genetic engineering advanced, scientists realized that a few specific types of bacteriophages could be turned into very useful research tools. One class of phages which began to be studied closely and intensely, belonged to a genus called *Inovirus*, which is in a larger family called *Inoviridae*. Phages in these groups have a combination of useful traits, which include the following:

(1) They do not have lipid envelopes, and instead are enclosed within "shells" (also called "capsids") made of protein subunits that are packed tightly together, comparable to bricks that form a wall. This is useful, since protein shells will not be damaged by various types of detergents which will attack and break apart the membranes that surround cells. Therefore, if an animal cell (or a "non-walled" bacterial cell) is treated with such a detergent, the cell will be broken apart ("lysed"), in a manner which will release the phages, with no damage to the phages. Those undamaged phages can then be reproduced, by using them to infect a new batch of host cells.

(2) The shells of *Inovirus* phages have cylindrical shapes; and, in most such phages, their diameters are very small (averaging about 7 nanometers, which is less than the diameter of a

single antibody molecule). Wild-type phages have lengths of about 900 nanometers, which is more than 100 times greater than their diameters, and they can bend and flex. Therefore, they often are called "filamentous" phages, with shapes comparable to tiny pieces of thread. This trait is useful, since filamentous phages can wriggle out of host bacterial cells, via "pores" which they create through the outer wall or membrane of a bacterial host cell, without having to kill and burst the host cell in order to escape. This is useful and valuable, since the still-living bacterial host cells can continue making large numbers of the desired phages, very rapidly. Viruses that can escape from their host cells, without having to "lyse" and break apart the host cell, are often called "temperate" phages.

(3) They carry single-stranded DNA (ssDNA), rather than RNA (as carried by some types of viruses). That is useful, because DNA is more stable and durable, and easier to work with, than RNA, under lab conditions.

(4) The strand of ssDNA is carried in a circular form, inside an *Inovirus* phage particle, with both ends of the strand linked to a DNA-handling enzyme called gp2. After that ssDNA circle is injected into a bacterial host cell, it duplicates itself, by creating a complete copy of the alternate (complementary, or anti-sense) DNA strand, which later becomes the "template" for making more strands of the phage DNA. In the normal reproduction process, the formation of the double stranded DNA briefly creates a circular dsDNA intermediate, which is then opened up and manipulated by the gp2 protein. Therefore, by using certain tricks, such as: (i) using phages with deleted gp2 genes, to transform bacterial cells; and, (ii) inserting a "plasmid origin of replication" into the phage genome, researchers were able to create engineered phages which can alternate – quickly, easily, and an infinite number of times – back and forth between dsDNA (plasmid) forms, and ssDNA (phage-packaged) forms, under controlled conditions.

(5) In addition, the genomes of *Inovirus* phages usually have DNA strands that are only a fraction (measured by length, or weight) of the DNA strands carried by most other types of phages. The widely used "M13" class of *Inovirus* phages has a wild-type genome containing only about 6400 bases (also referred to as 6.4 kilobases (kb)), while other phage types that are widely used in lab work (including lambda, T4, and T7 phages, as discussed below) have genomes more than 6 times larger, ranging from 39 to 160 kb. This allows *Inovirus* phages to reproduce *VERY* rapidly; indeed, when that very small genome is combined with an ability to escape from host cells without damaging the host cells (thereby allowing the host cells to continue making more phages), *Inovirus* phages may well be the fastest-replicating microbes that have ever been "discovered" on this planet (that term is distinct from "ever *created* on this planet"; there are genetically engineered and "turbo-charged" variants of T7 phages, discussed below, which may well rival *Inoviruses*, in how fast they can reproduce).

(6) The entire genome of each *Inovirus* phage is contained in a single continuous strand of DNA. That also is convenient, since some types of viruses contain multiple segments of DNA or RNA, and all of those segments must be handled and packaged together, to create viable and infective virus particles.

(7) In addition to all of the foregoing factors, the lengths of *Inovirus* DNA strands can be

increased, extensively, by putting large "foreign DNA inserts" into them, without damaging or crippling the resulting phages.

The last factor above merits attention, since it helps describe how these phages are made, as they emerge from a bacterial cell. When a strand of *Inovirus* phage DNA is ready to begin emerging from a cell, a cluster of specialized proteins (encoded by the phage DNA) is assembled, to create a "cap" which will become the "blunt end" (also called the tail end) of the phage. Next, thousands of small protein units are packed together, like bricks that are being used to create a tall cylindrical smokestack, in a helical pattern which creates a cylindrical shell that surrounds and encloses the circular loop of ssDNA (since the bricks overlap somewhat, they sometimes are compared to fish scales). That small brick-like protein is called "coat protein 8", which is abbreviated herein as cp8 (also referred to elsewhere by terms such as CP-8, cpVIII, etc.). It also is called the "major" coat protein, since there are far more copies of cp8 than any other *Inovirus* proteins (when viruses are discussed, the term "major protein" does not refer to the largest protein; instead, it refers to the protein with the highest number of copies).

That elongation (or "packaging") process continues, until the "tail end" of the DNA loop reaches the "pore" (which passes through the cell membrane) through which the phage is emerging, as it is being assembled. When the "tail end" of the phage DNA strand reaches the exit point, the assembly of cp8 protein units terminates, and a small cluster of different phage proteins is added to the end of the phage capsid. That cluster includes 5 copies of a long "cell grabbing" protein – called the cp3 protein herein, and also called CP-3, cpIII, etc., in various other sources – which enables a phage particle to "grab hold of" a new bacterial host cell, as one of the steps in infecting a new host cell, to make more copies of the phage.

While those five cp3 proteins can be compared to the fingers on a hand, a better analogy compares them to the tentacles of a squid which is hunting for food in the deep ocean, where there is no light. A hunting squid will extend all of its tentacles outward, in the hope that any one of them will bump into or brush against some type of prey, or might sense some sort of ripple in the water which will indicate that something is swimming, somewhere nearby. If even a single tentacle can use its suction cups to create an initial grip on something, that something might become food, and the squid will do whatever it can to "reel it in", bringing its other tentacles into play as part of the process. A phage uses its cp3 "cell grabbing" proteins in similar ways.

Furthermore, because of how cp3 proteins work, they evolved in ways that allow and even encourage them to stretch out and extend, as far as possible from the tip of the phage particle they are attached to, rather than rolling or coiling up into smaller, shorter clumps, spheres, helices, or other compacted shapes.

A detailed article (Smeal 2017), describing the *Inovirus* replication cycle, can be downloaded at no charge via www.sciencedirect.com/science/article/pii/S0042682216302239.

Most unaltered "wild-type" strains of *Inovirus* phage contain about 2,700 copies of the cp8 protein (i.e., the small inactive chunks that are assembled into the phage capsid, like bricks being used to build a cylindrical smokestack). However, the assembly process will continue to simply "grind away", until the "trailing end" of the ssDNA loop reaches the assembly site, just

inside the outer membrane of the host bacterial cell. Therefore, the number of cp8 copies can be altered, substantially and even greatly, to make phages which are either substantially shorter, or much longer, than wild-type strains. As a result, engineered phages can be made with foreign inserts that can be quite long, and can include multiple newly-added genes, such as genes which encode enzymes that will inactivate antibiotics, such as tetracycline or ampicillin; this allows "selectable marker" genes to be inserted into phages, which is very convenient for lab work. One of the major steps forward, in that work, was in Zacher 1980, which announced the creation of an fd variant called fd-tet, which carried, not just a single antibiotic resistance gene, but an entire antibiotic resistance gene complex, including a repressor component that kept the gene silent, and inactive, when it is not needed. The fd-tet phage became a major lab workhorse, during the decades that followed, even though it was later found to have certain types of instability, as described below in a section which describes the development, 20 years later, of an alternate construct called the "fth-1" construct (which, more than 20 years after *it* was announced, became the "starting point" phage to the creation of a subsequent generation of phages called "fd-MAD" constructs, as described below, where the "MAD" acronym refers to "mucosal antigen delivery").

Nearly all *Inovirus* phages that are actively used in research have been engineered to carry an antibiotic resistance gene, as a "selectable marker gene" which will allow only those host cells which contain complete and functional copies of the phages, to grow in the presence of the corresponding antibiotic; and, because that extra gene requires the DNA genome to be longer, the capsids which hold that DNA strand also must be longer. Most engineered *Inovirus* constructs usually contain a strand of ssDNA with about 9000 or more bases, rather than the 6400 bases carried by the wild-type phages, and the capsid usually requires about 3000 copies of the cp8 protein, rather than the 2700 copies carried by wild-type phages.

After genetic researchers realized that filamentous *Inovirus* phages have a combination of convenient and useful traits, they began working with those phages, and especially with a subgroup called "Ff" strains. Several "sub-strains" of Ff phages emerged as focal points for development, including strains called the M-13, f1, and fd strains. Each of those gained its own body of advocates, adherents, and developers, who began tweaking and engineering each of those three main strains, in different ways.

Two different examples were created by research teams led by Prof. George Smith at the University of Missouri; his most often-cited report (Smith 1985) became a "citation classic", cited by more than 4500 articles by other researchers doing related work, and in 2018, he won a Nobel Prize for his contributions to the science of phages, and phage libraries. In the first major set of work he reported, the long finger-like cp3 proteins in M13 phages (attached in 5 copies to one end of each phage particle, with over 400 amino acid residues in each protein) were modified. During that early work, the cp3 protein was presumed to have two major active "domains". First, the "distal" domain (near the outer tip of each long finger) presumably would bind to certain types of specific proteins (called "pilus" proteins) on various types of bacteria (including *E. coli*, the "laboratory workhorse" bacteria that is used in nearly all genetic

engineering labs around the world). The second crucial domain (which can be called “proximal”, “embedded”, or similar terms) is partially embedded in the phage particle, in a manner which “alerts” a phage particle when its cp3 proteins have grabbed hold of a new host, which it can then try to infect. As Smith reported in 1985, he had discovered, by then, that foreign protein sequences could be inserted into modified cp3 proteins, in at least some locations between the distal and proximal domains, since neither of those two crucial functions, of those two different domains, would be disrupted by inserting a modestly-sized foreign insert, in between them. Accordingly, that launched the use of engineered phages, as laboratory tools and reagents which could be used to accomplish various things more quickly, and less expensively, than by using other approaches.

Subsequently, it was realized that the cp3 protein has two different binding domains, commonly called the N1 and N2 domains, with flexible glycine-rich linkers between them, while the “proximal” domain, which attaches the protein to the phage capsid, is called the CT domain. These are illustrated in Russel 2004, “Introduction to Phage Biology and Phage Display”, which can be downloaded at no cost from www.researchgate.net/publication/ 267975174_Introduction_to_Phage_Biology_and_Phage_Display. Most published reports of cp3 inserts have reported peptide inserts at amino acid positions 1 (i.e., the amino terminus of the protein), 198 (not recommended, since it can lead to an unpaired cysteine residue which can cause problems), or 249 (within the glycine-rich linker between the N2 binding domain, and the CT phage attachment domain).

Another major paper by George Smith (and Valery Petrenko), in 1988, focused on the much smaller (but much more abundant) cp8 protein. By then, Smith’s research group had inserted a new gene into a strain of phages called “Ff” phages to encode a second, additional copy of the cp8 protein, under the control of its own gene promoter. By placing the newly-added gene under the control of an “inducible” gene promoter (with activity levels that can be controlled, by controlling the concentration of an “inducer” chemical in the culture media), researchers could create phages which carried both:

(1) a controllable and limited number (up to a few hundred copies) of altered cp8 proteins, with inserted foreign peptide sequences up to about 15 amino acids long, located on accessible outer surfaces of those “brick-like” proteins, distributed randomly along the entire lengths of the cylindrical shells of the phage particles; and,

(2) thousands of unaltered (“wild-type”) cp8 proteins, which would help ensure that the phage particles would be “packaged” efficiently and rapidly, even though some of the cp8 copies were carrying the “extra baggage” of foreign inserts.

Those engineered phages – carrying two different genes to encode two different versions of the cp8 protein, under the control of different gene promoters – came to be known as “f88” phages, where the two 8’s represent the two different cp8 genes. For complicated reasons that do not merit a digression herein, the M13 strain which Smith had previously used did not work well, when tested for cp8 protein inserts, so Smith & Petrenko shifted over to the fd strain, which Petrenko had available (more information on that and related topics is contained in a chapter by

Petrenko & Smith, "Vectors and Modes of Display", in *Phage Display In Biotechnology and Drug Discovery* (Sidhu & Geyer, editors, CRC Press, 2nd edition, 2015).

Using the steps described above (and various other enhancements), the M13, F1, and Fd strains of filamentous *Inovirus* phages were modified and engineered to a point where they became "laboratory workhorses" that are used in genetic engineering labs around the world. Any well-known laboratory manual which describes genetic engineering methods and recipes will contain one or more chapters devoted to the use of filamentous phages. The most highly-respected and widely-used such manual today (and for the past 40 years) is *Molecular Cloning: A Laboratory Manual*, originally edited by Maniatis, Fritsch & Sambrook (now in its 4th edition, edited by Green and Sambrook, CSHL Press); it contains several chapters devoted entirely to how to make and use phages, as research tools. In addition, as a "spinoff" work, the same publisher has issued an entire laboratory manual entirely devoted to using phages (*Phage Display: A Laboratory Manual* (Barbas et al, CSHL Press, 2004)). A more recent competing laboratory manual also is available, *Phage Display: Methods and Protocols* (Hust & Lim, Springer/Humana Press, 2018). The book cited above, by Sidhu & Geyer, is not so much of a "hyper-technical, detailed recipes" treatment; instead, it has a narrative style that seems intended for lab technicians who are beginning a career in working with such phages. Another book worth noting is *Biotechnological Applications of Phage and Phage-Derived Proteins* (Santos & Azeredo, MDPI Press, 2019), a collection of articles and reviews. Those who prefer review articles, over full-length books and/or laboratory manuals, should be aware of Cabilly 1999 ("The basic structure of filamentous phage and its use in the display of combinatorial peptide libraries") and Rakonjac 2011 ("Filamentous Bacteriophage: Biology, Phage Display and Nanotechnology Applications"; a complete copy is available for download at no charge, via pubmed.ncbi.nlm.nih.gov).

It also should be noted that filamentous phages can infect only certain types of bacteria; they cannot infect mammalian or plant cells. Therefore, they generally are regarded and treated as non-pathogenic, and they can be used without expensive and cumbersome extra precautions that are required when pathogenic microbes are involved.

Any of various candidate strains of *E. coli* cells can be used with phages derived from the *Inovirus* group (including M13, f1, and fd phages), and many of these strains of host cells have various traits that can make them especially useful and well-suited for various types of research. Extensive information about any *E. coli* strain that is described in the literature can be downloaded at no cost from the "Coli Genetics Stock Center", an information resource run by a group at Yale University (cgsc.biology.yale.edu). As just one example, a strain called MC-1061 strain is often used, because it has a combination of two useful traits:

(1) The MC-1061 strain has a mutation (often called DH5α-, pronounced as DH5-alpha-minus) which prevents it from creating the "pilus" conjugation tubes which DH5α+ (alpha-plus) strains use to transfer genes to other bacteria. That trait causes it to be classified as a "female" strain, and female strains tend to be more genetically stable than "male" strains. Since f-type phages normally grab hold of *E. coli* host cells via a "pilus" protein which is expressed only by

"male" bacteria (and which is not expressed by MC-1061 cells), this creates an inconvenience, since f88 phages must be transfected into "female" MC-1061 cells by using methods normally used to insert plasmids into host cells (usually involving calcium chloride). However, that inconvenience can avoid potentially major complications, which can arise unexpectedly, and with no notice, and which can thereafter introduce major problems into certain types of analyses. Accordingly, the modest inconvenience of using a "female" strain is often regarded as being worth an extra step during transfection.

(2) The MC-1061 strain also lacks an enzyme in the so-called "lac repressor" system. For reasons discussed in more detail below, most *E. coli* cells do not normally express or contain certain enzymes that enable the cells to metabolize, break apart, and utilize a specific sugar molecule called lactose, as a carbon source, so long as other types of more common sugars are available. Under normal conditions, the absence of unused lactose-metabolizing enzymes allows such cells to grow more rapidly, compared to cells which must "carry that extra baggage". However, in the absence of glucose or other common sugars, the lactate enzyme "repressor" system is inactivated, and enzymes which can metabolize lactose begin to be expressed. The discovery of how that system works (which led to a Nobel Prize, in 1965) led to the creation of "lactase promoters" (and even more sophisticated versions, called "ptac" promoters) as "inducible" promoters, in genetic engineering.

The phrase "inducible promoter" refers to a gene promoter which normally will stay silent, unless and until an "inducer" compound is added to the culture medium which contains the host bacteria (or, until some other controllable factor is altered). In the case of lac and ptac promoters, the most widely used inducer chemical is abbreviated as IPTG (the full name can be found easily on the internet). Accordingly, in a host bacterial strain (such as the MC-1061 strain) in which the "lac repressor" system has been deactivated or removed, an inserted foreign gene which is under the control of the lac or ptac promoter will always be functioning, at some modest level (such genes are usually called "constitutive genes", to distinguish them from "inducible" genes). Nevertheless, if quantities of IPTG are added to the culture media which holds the host bacteria, then the quantities of proteins expressed by those genes will still increase, to higher levels. As a result, strains such as MC-1061, in which the "lac repressor" system is missing or inactivated, can be very useful for research involving phages carrying inducible promoters.

Another highly important and widely-used class of *E. coli* cells that are used to grow *Inovirus* phages is called the K12 class. As described in more detail below, it is a "hobbled" strain, which can grow and reproduce, without needing special nutrients to be added to the nutrient broth or agar; but, it can reproduce only very slowly, unless those special nutrients are added to the broth or agar. Therefore, it cannot compete effectively against wild-type strains, so it is regarded as a generally harmless host; and, when used for industrial-scale fermentation, it can be pushed in a direction where the cells will expend essentially all of their available energy, and resources, creating large amounts of a specific protein which that process is manufacturing, while creating only very small numbers of new cells, which are not the desired product.

There are inevitable concerns over whether any new types of phages might cause

digestive problems, if they begin to affect and alter the "bacterial flora" mixture that forms a crucial part of the digestive process, inside the intestines of any animal. However, two powerful reassuring factors come into play. One is that these types of *Inovirus* phages have been colonizing, co-existing, and co-evolving with entirely normal and healthy intestinal bacteria, for many thousands of years. The second is that the specific strains of *Inovirus* which have been converted into "laboratory workhorses", in molecular biology and genetic engineering labs, have been largely "disarmed", in various ways, and usually need to be paired up with a specific known "co-engineered" strain of host bacterial cells, which will provide special nutrients or other conditions that their paired-up phages need, to grow and reproduce.

Nevertheless, despite the two reassuring factors above, regulatory agencies in all industrial countries (such as the Food and Drug Administration, in the US) actively review and monitor any proposed use of phages, in any medical or nutritional products or processes.

As a final comment on filamentous (and other) phages, the phrase "phage display" has become a shorthand reference to any type of phage which carries any foreign polypeptide segment, at a location where the foreign polypeptide will be surface-exposed and readily accessible. That phrase is not always used consistently; it can be used as a noun which refers to a single particle, or as a noun which refers to a population or preparation of such particles, or as an adjective (such as in the phrases, "phage display library" and "phage display vector").

OTHER (NON-FILAMENTOUS) PHAGES USED IN LABS

In addition to the M13, Ff, and Fd classes of phages (all of which are "strains" of filamentous *Inovirus* phages), several other types of phages should be noted, since they also are widely used in molecular biology research. Since no research team would want to put serious and sustained effort into enhancing, engineering, and tweaking some phage type which merely does the same things that other available engineered phages can already do, it should not be surprising that each of these three additional classes of phages have been optimized to fill some particular niche, or type of use. Accordingly, the most widely used types of non-filamentous phages are:

(1) lambda phages, which have an enlarged "head" region which carries a surface-mounted protein which – since it generally points outwardly, instead of having to be packed together tightly – can carry far more copies of large and long foreign polypeptide inserts, compared to filamentous phages (which can carry foreign inserts up to only about 15 amino acids long, in their brick-like cp8 proteins, and which have only 5 copies of the finger-like cp3 proteins). Since lambda phage coat proteins are present in about 400 copies per particle, they can carry about 80 times as many copies of a foreign insert longer than 15 amino acids, compared to using cp3 proteins in filamentous phages.

On the negative side, the wild-type genomes of lambda phages are about 50 kilobases (about 8 times larger/longer than *Inovirus* phages, with 6.4 kb); and, lambda genomes carry double-stranded DNA, rather than the ssDNA carried by filamentous phages. Also, their enlarged "heads" cannot escape through pores in bacterial cell membranes; therefore, they must kill and burst open ("lyse") their host cells, to escape. Those factors lead to slower reproduction

and fermentation rates, compared to *Inovirus* phages; however, lambda phages still can reproduce far more rapidly than bacterial cells. Therefore, if the main "incremental cost" of using lambda phages (rather than filamentous phages) is a requirement to ferment them for an extra day (using bacterial nutrients that are relatively inexpensive), or in somewhat larger tanks (which do not need to endure high pressures or corrosive conditions, and which therefore can be made at reasonable cost), such costs become relatively minor and tolerable, if a project needs foreign inserts which are larger than can be created by the cp8 proteins of filamentous phages.

(2) T4 phages carry dsDNA, with a genome of about 160 kilobases (i.e., about 25 times longer than filamentous phages). They are not well-suited for use as potential vaccine particles, because they are among the largest and most complicated phages known to exist, with very large genomes, and very large numbers of different proteins, any of which might trigger the formation of useless antibodies which will bind to those proteins, rather than to a desired and useful "payload antigen" sequence; nevertheless, they merit a mention in any overview of lytic phages, due to both:

(a) their historical importance. Beginning in the 1940s, many of the most important discoveries in cellular biology and nucleic acids came from studies of how T4 phages interacted with *E. coli* bacteria. Therefore, by the time the 1970s and genetic engineering arrived, an enormous knowledge base was already present, centering on T4 phages; and,

(b) a fact of biology: T4 phages are exceptionally aggressive and fast-reproducing. The amount of time that passes, from the moment a single T4 phage enters an *E. coli* cell, until the cell bursts open (releasing dozens or hundreds of new copies of the T4 phage) is only about 30 minutes (which also can be achieved by T7 phages, discussed below).

It also should be pointed out that, while the "T-numbered" phages (such as T1, T2, etc., up through T7) were assigned numbers in the order they were discovered, it turned out (purely through coincidence) that the T2, T4, and T6 phages are very similar to each other, in their structures and genomes. Because those are the three even-numbered phage groups, they often are referred to collectively as the "T-even" phages, and any general comments about any member of that class usually also will apply to other T-even phages, as well.

It also should be noted that all of the "T-even" phages carry genes which encode more than 100 proteins, in any of those phages. This points away from using any of them as vaccine vectors, since unhelpful antibody-forming responses might be generated against numerous such proteins, in ways that would both detract from, and divert useful resources away from, desirable antibody responses to pathogen-derived antigenic sequences inserted into such vectors.

(3) T7 phages also have dsDNA genomes (about 39 kb in length), and wild-type T7 phages have a capsid protein which is produced in two different forms (the 10A version, with 344 amino acids, and the 10B version, with 397 amino acids). The 10A/B gene can be modified, to create both: (i) large surface-mounted foreign peptides, in relatively low copy numbers; and, (ii) smaller surface-mounted foreign peptides, in roughly 10-fold higher copy numbers. Accordingly, this phage can be used, if desired, to create vaccine particles which can carry both: (i) a first peptide sequence, in fairly low copy numbers (such as about 40 copies/particle), as part

of the 10B proteins; and, (ii) a second peptide sequence, in roughly 400 copies per particle, as part of the 10A proteins. In addition, a number of strategies and tricks have been developed (which are described in more detail, below) which can be used to make T7 phages easier to work with, such as by: (i) using a small and workable "helper plasmid" with an "essential metabolite" gene, as well as an engineered 10A/B gene, to transfect *E. coli* cells which cannot make that essential metabolite; (ii) infecting those host cells with wild-type T7 phages; and, (iii) using selection methods to isolate modified T7 phages which were created by a natural DNA-swapping process called "homologous recombination", and which will carry both the engineered 10A/B gene, and the essential metabolite gene, within a complete and engineered T7 genome that can make unlimited numbers of the desired types of candidate vaccine particles, carrying both a MALT-targeting sequence in limited numbers, and a selected antigen in much larger numbers. Because of those and other factors, as described below, T7 phages were chosen as the main initial "vehicle" for creating vaccine particles which will be used in the first "pathogen challenge tests", which will evaluate the efficacy of those candidate particles in actually protecting inoculated animals against a known pathogen. Those T7 constructs, and T7 "cassettes" that have been designed and created to make it easy to delete their current antigen gene sequence, and replace that antigen sequence with a short and inexpensive alternate DNA sequence which will encode any other antigen protein sequence which is of interest to any research team, are described in more detail below, since they are part of this invention.

(4) A recently discovered class of phages, called AP205, is one of a class of phages that infect bacteria called *Acinetobacter*. Those bacteria have been known for years mainly as a type of soil bacteria, which can also become opportunistic pathogens. They became of serious medical and research interest after soldiers who were in Iraq, or who had returned from Iraq, began suffering from a variety of severe medical problems, relating to antibiotic-resistant strains of *Acinetobacter baumannii* (which picked up the nickname "Iraqibacter"). As a result, phages which specifically attack and kill those bacteria were pushed into the forefront of treatments against those bacteria, largely due to the actions of an epidemiologist, Steffanie Strathdee, who (as mentioned above) pushed the medical authorities, hard, to use them, to save her husband's life. A comparative analysis of 37 known types of phages that can attack *Acinetobacter* is provided in Turner 2014.

A specific strain of phages, which came to be known as AP205 phages, was discovered to have some unusual traits; as described in Shishovs 2016, it "has a coat protein sequence not similar to any other known single-stranded RNA phage." When they analyzed it, they realized that both the N-terminus, and the C terminus, of its major coat protein are exposed on the surfaces of the phage, and either or both of them can tolerate relatively long foreign add-on peptide sequences. That made them interesting and useful, and a number of research teams have begun using them, especially since they are well-suited for use with another recent development, called the "Spy-Tag/SpyCatcher" (or its shorter form, "Tag/Catcher") system. That system, described in articles such as Thrane 2016 and Brune 2016, enables cross-linking of two proteins to each other, in a manner which creates strong covalent bonds (rather than merely "affinity-

bonded couplings", as occur between antibodies and antigens, or between avidin and biotin, which are widely used as coupling agents, in research). Accordingly, either the Tag sequence, or the Catcher sequence, can be added to a surface protein on a phage, and the other sequence can be added to any antigen (or other) sequence of interest, to enable the antigen or other foreign sequence to be attached to the phage (and, to allow simple and easy coupling of unlimited numbers of candidate sequences, to any phage particle having either a Tag sequence, or a Catcher sequence, engineered into its surface proteins).

All four types of phages mentioned above (i.e., lambda, T4, T7, and AP205 phages) have generally spherical "head" components. While that shape forces them to act as "lytic" bacteria, which need to kill a host cell to escape from it, it has an offsetting benefit. That shape renders lytic phages, including all four types listed above, much better suited than filamentous phages, for an important type of research which uses automated machines to do high-speed analysis and sorting of particles, depending on whether they have been labeled with fluorescent "reporter" molecules. As described below, that process initially was called "flow cytometry", since it uses continuous flow, to analyze cells. When scientists figured out how to successfully adapt it for use with viruses with generally spherical heads, starting in about 2013, they called that new set of processes "flow virometry".

EARLY WORK TO DEVELOP PHAGES INTO RESEARCH TOOLS

This section discusses some of the work that was done from about 1970 to 2000, to modify several selected types of phages to a point where they would provide useful tools, in molecular biology and genetic engineering labs.

Important early work in this field was done by Prof. George Smith (mentioned above), which included a seminal 1985 article entitled, "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface." That (and additional) work by Smith and his team led to two sets of results worth noting in this section.

One result was the disclosure of faster, easier, better, and cheaper ways to make protein segments up to about 15 amino acids long, in large numbers (up to several hundred copies per particle), on the surfaces of the cp8 coat protein in "engineered" phages.

Those types of engineered phages, carrying known foreign polypeptide inserts, can be used in various ways. As just one example, such phages can be injected into lab animals, to initiate an antibody-forming response, leading to "B cells" making antibodies which will bind to various protein sequences on the surfaces of the phage particles. B cells which happen to make antibodies which bind to the foreign insert can be identified, and isolated. Using special mild detergents to weaken (but not break) the cell membranes, to a point where two cells will coalesce into a single larger cell, B cells which secrete the desired antibodies can be "fused" with cancerous (or "transformed") cell lines, which can be grown endlessly, in lab conditions. After the merged cells stabilize (which involves discarding some of their surplus chromosomes), the resulting merged cells can then be screened, to identify and isolate those particular cell lines which happened to receive both: (i) the "oncogenes" (from the cancerous cells) which will allow

the merged/fused cells to continue growing and reproducing indefinitely; and, (ii) the antibody-encoding genes (from the B cells) which will express the desired types of antibodies. The "hybridoma" cells which meet those criteria can then be used to create an unlimited supply of "monoclonal antibodies", which will bind to any proteins that contain, on any accessible surfaces, the polypeptide sequence which was inserted into the phages at the start of the process. This type of work is discussed in more detail in Alfaleh 2020, a review article entitled, "Phage Display Derived Monoclonal Antibodies: From Bench to Bedside".

Therefore, the types of phages developed by Smith and his coworkers can be injected into animals, to trigger the development of B cells which will create antibodies which bind to the foreign antigen sequence on the injected phages; and, those B cells can then be used to create hybridoma cells, which will secrete monoclonal antibodies which will bind to the protein sequence that was inserted into the cp8 coat protein, on the phages that were injected into the animals.

A second major useful result created by Smith involved useful and effective ways to create something called a "phage display library". Like a conventional library, which can hold millions of different books – with a different text, in each different book – a "phage library" can contain billions of different phages (indeed, one of the most commonly purchased libraries contains about a trillion different phage particles, all carried within in a single small and convenient tube). Each phage will carry, in a specific segment of a specific gene, a totally random set of DNA bases, created by randomized chemical synthesis methods. All of the phages in a library will be identical, except for that one short segment of DNA, and the corresponding short segment of amino acids in the protein encoded by that gene. The most commonly and widely used phage display libraries are made from *Inovirus* phages (i.e., the types of filamentous phages that are described in some detail above, which include M13 and fd phages); and the randomly-generated DNA inserts are located at a precise location in the cp3 gene, which will cause the peptide segments which those randomized DNA sequences encode, to appear at the outermost tips of the long "cell-grabbing" cp3 proteins, which are present in 5 copies on each phage particle.

Accordingly, a "phage display library" can have billions or even trillions of phages, all contained within a 1 or 2 milliliter volume, and each phage will carry a random peptide sequence, in one or more exposed locations on the surfaces of the phages.

Those types of libraries are normally used in complex and carefully-designed research projects which use "screening tests", to expose millions of phages at a time, in a few tiny droplets of liquid, to some carefully chosen and prepared set of cells, or pieces of tissue, or collection of antibodies, or targeted organs in the bodies of small animals, or other biological preparation, in order to determine which particular candidate phages happened to cling to, or be taken in by, or otherwise react with, or interact with, the cells, tissues, antibodies or other biological materials that were used in that particular screening test. Using any of numerous possible (and often entirely new, and innovative) screening methods, the particular "winning candidates" (among the millions of "contestant" phages) which perform in some desired way, in

some particular screening test, can be isolated. The small inserted foreign amino acid sequences which were added to the "winning" phages can be processed, to determine their foreign DNA and amino acid sequences; and, that newly-discovered information can then be used, by the researchers, in any way they desire.

That type of screening test – using a well-known (purchased, and readily available to anyone) library (made from *Inovirus* phages having random foreign inserts at the tips of their cp3 proteins) – is exactly what was done, to isolate and then analyze the "targeted transport" sequences that are discussed in the Summary and Detailed Description sections, below, which describe this invention; however, it must be emphasized that that "type" of screening test actually involved a brand new, innovative, never-before-used set of steps, to isolate a class of peptides which had never previously been isolated by any other screening tests. Those peptides became the "MALT-targeting" peptides which are described in detail below, and which are part of this invention.

Some of the early work to create "phage display libraries" used the cp8 genes and proteins, of *Inovirus* phages, to receive and carry the foreign inserts. Those constructs came to be called either:

(a) "f88" constructs, if the first, original, "wild-type" cp8 gene – as well as the modified "second" cp8 gene, carrying the insert – were both contained in the phage genome; or,

(b) "f8+8" constructs, if one of the cp8 genes was contained in the phage genome, but the other cp8 gene was contained in a separate genetic construct, which in nearly all cases would be called a "helper plasmid" or a "helper phage".

As described below, researchers gradually realized that it was not necessary to have 100 or more copies, of a foreign insert, on the surface of each and every one of the billion or more phage candidates, in a phage display library; and, therefore, instead of needing cp8 libraries that were created by "f88" or "f8+8" phage constructs, researchers could use cp3 libraries, instead, which would carry 5 copies of each foreign insert, out near the outer tips of the long tentacle-like cp3 proteins. Cp3 libraries were easier to make, easier to maintain, and entirely adequate for the types of screening tests they were being used in; and so, cp8 libraries fell out of favor, and ceased to be used, or made, or maintained. Cp3 libraries took over; they became the standard types of phage display libraries, and they continue to be improved, and sold, today.

Many types of "screening tests" (described above) suffer from high levels of unwanted "noise", created by non-specific binding of phages (which tend to be relatively "sticky") to mammalian or other cells of interest. This led to the development of a more elaborate process, called "biopanning" or simply "panning" (the word was derived from the process that miners use to identify and isolate nuggets of gold, silver, or other precious metals, from large amounts of dirt and rock). It is described in articles such as McGuire 2009, entitled, "Biopanning of Phage Displayed Peptide Libraries for the Isolation of Cell-Specific Ligands." One noteworthy quote from that article is, "Selection of a peptide ligand, using our protocol, should be expected to take 5–6 rounds of biopanning." Anyone interested in that process can find more information in that and other similar articles.

Regardless of which particular process is used, most types of screening or panning processes can be arranged and performed so that the "winning phages" will be fully viable, and able to infect (and reproduce in) new host bacterial cells, to form any desired quantity of the "winning" phages, with all of those "clonal" copies having exactly the same foreign inserts. One of the key factors which enables that, is that cells are surrounded by membranes, made from "lipid bilayers", while the types of phages that are used to make display libraries are "non-enveloped" phages, which do not have membranes, and which are surrounded, instead, by protein subunits which are packed together tightly. As a result, various mild detergents have been identified, which can break apart the membranes of cells, without affecting the proteins that enclose the phages.

And, after a "winning phage" has been isolated, the DNA sequence (and amino acid sequence) of the foreign insert can be determined, and those exact same sequences can be inserted into any plasmid, phage, vaccine particle, or other construct. Accordingly, those types of screening and/or panning tests, using "phage display libraries" as created by Smith and other researchers, are highly useful in biological and medical research.

PRIOR ART PROPOSALS FOR PHAGES AS VACCINE PARTICLES

Beginning in about 2000, research articles began to propose that phage particles could be used to carry antigenic proteins, in ways that might make them useful as vaccines. Such articles include, for example, Clark 2004 ("Bacterial viruses as human vaccines?"), Jafari 2015 ("Phage Particles as Vaccine Delivery Vehicles: Concepts, Applications and Prospects"), Aghebati-Maleki 2016 ("Phage display as a promising approach for vaccine development"), and Bao 2019 ("Phage-based vaccines"). Hess 2019 ("Phage display as a tool for vaccine and immunotherapy development") is a well-organized and recommended review, and a full copy can be downloaded at no cost from www.ncbi.nlm.nih.gov/pmc/articles/PMC6971447.

The reports cited in Hess 2019 include, for example:

(i) Bahadir 2011, in which antigens from hepatitis B viruses were placed on M13 phages, and used to inoculate mice;

(ii) Gao 2011, in which antigens from Epstein-Barr viruses were placed in T7 phages, and used to inoculate rats; and

(iii) Xu 2017, in which antigens from foot-and-mouth disease viruses were placed in T7 phages, and used to inoculate pigs.

All three of those reports indicated that antibodies were formed, in the inoculated animals, which would bind to the spliced-on antigens. However, to the best of the Inventor's knowledge and belief, as this is being written:

(i) none of those articles has led to any type of usable product, and no phage-derived vaccines have ever been approved, for medical, livestock, or veterinary use, in any industrialized country; and,

(ii) none of those efforts involved any attempt to identify or use any sort of "targeted transport" peptide, to create vaccine particles that would be more potent, and more effective.

Other review articles have appeared, describing various efforts which began in 2020 or later, to develop vaccines against corona viruses, mainly focusing on the class of corona viruses which triggered the "COVID" pandemic which started in late 2019, and caused at least 3 million deaths in 2020, before mRNA vaccines began to become available in December 2020. Palma 2023 (which was limited to a literature review, and did not describe any new research by the author; a full copy can be downloaded via www.ncbi.nlm.nih.gov/pmc/articles/PMC9967953) reviews and cites a number of articles describing that research (Tables 1 and 2 both contain extensive lists of such articles, with hyper-linked footnotes, and many of the footnoted references also contain hyperlinks which can take a reader directly to more information about that article). As just one example, Davenport 2022 described how antigens from the SARS-CoV-2 and MERS-CoV viruses were added to "phage-like particles" derived from lambda phages, and were injected into mice.

To the best of the Inventor's knowledge and belief, the only article cited by Palma (with a description in the text, pointing out the unusual nature of that article) which related in any way to phage particles which also contained "a transport peptide, added to the particles," was the Staquicini 2021B/PNAS article (Palma's footnote 92).

If someone goes digging into that Staquicini 2021B/PNAS article, they will soon discover that it is one of two paired research articles – cited herein as Staquicini 2021A/MedNY and Staquicini 2021B/PNAS – which require sustained and careful attention, by anyone interested in this field. Both of those articles listed 21 co-authors, and the first (and several other) authors were the same; however, the majority of authors, in those two articles, were different.

Staquicini 2021A/MedNY described the isolation – by screening a phage display library – of several transport sequences that could transport particles into circulating blood, by gripping certain types of "integrin" receptors on certain types of cells in the lungs. When that receptor-gripping reaction occurred, those lung cells pulled those particles inside the cell, and then passed them through the lung cell, into circulating blood. That was shown, by mounting those transport sequences onto cp3 proteins of *Inovirus* phages, and administered droplets containing those phage constructs, into the lungs of mice, and later into macaque monkeys, by having the animals breathe aerosols containing those droplets. Those phages triggered the formation of both IgG antibodies, and IgA *monomers*, which were then found in the blood serum of the treated animals. They had *no reason to expect* that those phage particles, upon reaching the bloodstream, would lead to *mucosal* immune responses, and they apparently *never analyzed any mucosal fluids*, to find out whether any IgA dimers were created. So, their method offers another way to get antigens into circulating blood, to trigger the same types of internal antibody responses that are triggered by conventional injected vaccines, without having to use needles, or injections. It did not teach or suggest any way to create mucosal immune responses, or secreted IgA antibody dimers in mucosal fluids.

Staquicini 2001B/PNAS described subsequent phage constructs, which contained the same transport sequences, in the same cp3 proteins, and which also contained several antigenic sequences (one such sequence, per phage) derived from corona-virus "spike" (S) proteins,

mounted on cp8 proteins. When they administered those phages to the mice and monkeys, again via aerosol inhalation, the phages again entered the blood of the animals, and the researchers were able to detect both IgG antibodies, and IgA antibody monomers, in the blood, which would affinity-bind to the S protein sequences. As in the Staquicini 2001A article, they apparently never tested any saliva, or any other mucosal fluids; accordingly, neither of those two articles taught, or suggested, any way to create a mucosal response, which would create secreted IgA antibody dimers in mucosal fluids. Instead, both articles merely described another route for creating the same types of internal immune responses that lead to IgG formation, without having to use needles, or injections.

That work is interesting, and relevant; among other things, it helps to confirm and support the potential of creating phage particles which contain transport sequences added to one protein, and antigen sequences added to a different protein. However, it only reported another pathway for getting candidate vaccine particles into circulating blood, and plenty of other methods for doing that same thing are already known.

It also is worth noting that a number of the authors of those two papers created a company, called PhageNova Bio, presumably to try to commercialize their discovery, and they also filed PCT patent application PCT/US2020/053758, which claims the specific trans-lung targeted peptides they discovered, and various methods of using those peptides. The invention herein does not infringe, or overlap with, or need to take any steps or precautions to avoid infringing, any of those claims.

The Ferguson 2008/2010 Prior Art (By the Same Inventor Herein)

Another item complicates the prior art in this case, and it requires careful attention. It is cited herein as "Ferguson 2008/2010", since it arises from two published applications having the same content. The Patent Cooperation Treaty version was published as WO-2008/148164, and the US version was published as US 2010/0278846. Both were entitled "Nasal-Administered Vaccines Using Multi-Screened NALT-Targeting and Phagocytic Polypeptide Transport Sequences." Both were by the same inventor named herein, and both were written by the same patent attorney who wrote this application.

An adequate understanding of that item of prior art requires it to be seen and considered from at least two different perspectives. Since any building, animal, or complicated statue can have multiple different appearances, depending on whether one looks at it from the front, the side, or some other angle, one needs to recognize and accept that every perspective can add an additional dimension, layer, and component to a viewer's overall understanding and grasp of what that building, animal, or statue actually looks like. In much the same way, the Ferguson 2008/2010 prior art needs to be recognized as both: (i) an essential and absolutely necessary learning experience, process, and progression, which led to the successful second attempt; and, (ii) a failure, which contained certain statements, claims, assertions, and predictions which, although made in complete good faith when they were written, were later discovered to be untrue, unreliable, and in need of correction, based on what was later discovered.

Two examples of statements which were made in good faith at that time, but which were later shown to be inaccurate and untrue – by research which was performed only after those applications were published – are as follows:

From the Summary of Invention: "The coat proteins of phage vaccines for nasal usage must contain foreign polypeptide sequences that will cause the phage particles to bind to and activate nasopharyngeal-associated lymphoid tissue (NALT) cells. *Such phages have been . . . identified and isolated from a phage display library . . .*"

Claim 6: "The vaccine preparation of claim 1, *wherein said virus particles have been shown by screening tests to be taken into nasopharyngeal-associated lymphoid tissue cells* and subsequently taken into phagocytic antigen-presenting cells."

The italicized portions of both of those statements were later found to be unreliable, and false, and therefore, they are hereby directly and explicitly disclaimed, disavowed, corrected, and superseded by the information herein, for the following reasons.

Subsequent research – as described and explained in Example 20, below, which was performed after the 2008/2010 publications appeared – completely failed to show that the particles identified by the 2008/2010 screening processes were, in fact, being taken in by NALT patches, or by any other type of MALT patches. Instead of proving that the desired types of MALT-targeting transport sequences "have been shown" to perform as desired, the results of that subsequent research strongly indicated, instead, that the phage particles carrying the only "purported MALT-targeting transport sequence" which was actually tested (which had shown up in 39 of the 42 clonal phage isolates that were created by the screening tests used in the 2008/2010 publications) was being taken up by *NON*-NALT "subepithelial" layers and cells, just below the surfaces of the nasal airway membranes, in ways which apparently would not – and could not – lead to the formation of secreted mucosal IgA antibody *dimers*, which are the primary and essential component, and indicator, of a truly effective mucosal immune response. The phage particles having that "purported MALT-targeting transport sequence", identified by the screening process described in the 2008/2010 publications, were transported to conventional internal lymph nodes, rather than to MALT tissue patches; and, when that happens, the types of types of antibodies which will be created are the same types of conventional Y-shaped IgG antibodies that are formed in response to conventional injected vaccines, *rather than* the types of secreted IgA dimers which indicate a successful mucosal immune response to an effective mucosal vaccine.

Furthermore, since that post-2010 research (as described in Example 20) gave every indication that the "purported MALT-targeting transport sequence" could not actually perform as hoped (i.e., by triggering the production and secretion of IgA antibody dimers, into saliva), they were never even tested, to see whether they could trigger IgA production. Instead, the repeated failures of those phages to show any indication of uptake and/or processing, by actual and true MALT tissues, led to a complete "reboot" and restart of the project described herein.

When those repeated failures kept occurring, they led gradually to the assumption, realization, and conclusion that the old cp8 display library which was being used – which had

been screened, to obtain the purported MALT-targeting sequences reported in the 2008/2010 publications – had been hopelessly overwhelmed and overgrown by the specific phage which carried that particular insert. As a result, a decision was made to completely abandon and stop trying to work with that old cp8 library, and to begin again, and begin anew, with a newly-purchased cp3 library.

That turning point led to the creation and development, by the Inventor herein, initially of a first type of screening test, and then of an even more powerful, selective, and revealing second type of screening test, where both of those new screening tests used and exploited the surface receptors on dendritic cells (as described below, in the Detailed Description section). Those two screening methods were completely new and innovative, and were not anticipated in any way by anything described in the 2008/2010 publications; and, those new screening tests (combined with several other factors, including the super-thin, super-precise cell-harvesting tool described below) were what enabled the new invention, as described herein, to succeed.

"ADJUVANTS" WHICH CAN MAKE VACCINES MORE POTENT

The term "adjuvant" comes from a Greek root which means "to help". A starting-point definition, provided in Wu 2021 (a review article entitled, "Overview of Vaccine Adjuvants"), is, "Adjuvants are defined as materials added to vaccines in order to improve the immunological response." That is a good and reasonable starting point, but a clarifier to that statement should be added, to require that an adjuvant must be actively "immuno-stimulatory", to a level which must be greater than various merely "supportive" additives, which are classified as "excipients", a pharmaceutical term which refers to compounds that can improve the performance of the active ingredient(s), in a preparation containing that ingredient. Examples of excipients include diluting or liquefying agents, binders, emulsifiers, preservatives, etc.

***NOTE ADDED RE: A CRUCIAL FACTOR, NOT IN PATENT APPLICATION*:**

As we continued to do more work, we gradually came to realize that scientists and physicians adopted the term "immuno-stimulatory", for adjuvants added to injected vaccines, to distract attention away from a highly unpleasant fact that they would prefer to not be confronted by, or to discuss publicly. The sad fact is that adjuvants are "necessarily nasty" compounds. They are harsh, irritating, inflammatory, distress-causing (and therefore "noxious" and even "toxic") compounds, which are added to injectable vaccines, in order to cause deliberate distress and discomfort to the muscle cells, at the injection site. Why? To force those distressed muscle cells to send out distress signals (in the form of messenger molecules, which are called "cytokines" rather than "hormones" when the immune system is involved). Those distress signals will recruit any nearby immune cells to come to the injection site, ***as quickly as possible*** – which is important and even essential, for ***injected*** vaccines, because the immune cells need to reach and begin processing the vaccine particles as quickly as possible, *before* the injected vaccine particles can be degraded, diluted, or diffused by normal and natural cellular and tissue repair mechanisms. Therefore, "adjuvants" are indeed "immuno-stimulatory", *BECAUSE* they are harsh, irritating, inflammatory, distress-causing additives. This factor is so important that, if MALT-targeting vaccines are able to eliminate the need for adding adjuvants to vaccines, the "new generation" of vaccines may end up being called "***post-adjuvant*** vaccines".

There are numerous review articles which discuss vaccine adjuvants in detail, and which usually divide them into various categories, based on their mode of action; examples include de Veer 2011, Alving 2012, Shah 2017, Wu 2021, and Firdaus 2022. Without getting too deeply into those issues, experts often use either or both of two phrases to describe the mechanism(s) of how adjuvants work. Those two phrases are "the danger signal theory", and "the pattern recognition theory". Briefly, "the danger signal theory" basically says that if a vaccine is accompanied by essentially any type of adjuvant which functions as an irritant and which provokes inflammation, swelling, or similar responses, mobile immune cells will be attracted (by messenger molecules which play the same role as hormones, but which are called cytokines, rather than hormones, when immune cells are involved) to the site where the vaccine was injected, and that will lead to a stronger, more potent response to the vaccine. The most widely used adjuvant in this category is called alum, a sulfate salt of aluminum.

By contrast, the "pattern recognition" theory says that hundreds of millions of years of evolution have provided mammalian immune systems with sophisticated mechanisms for recognizing when important invasive threats have breached the outer defenses, and must now be dealt with by the immune system, and, therefore, any really good scientific search for better adjuvants should focus on things like "pathogen-associated molecular patterns" (abbreviated as PAMPs). One of the most widely used agents in this class contains a type of semi-bacterial cell, called mycobacteria; the "myco-" prefix is the Greek root for fungus, and cells in the mycobacteria class have traits that put them into a category somewhere between bacteria, and fungus. They are pathogens, and cause diseases such as tuberculosis and leprosy, so they must be thoroughly killed before being added to an adjuvant mixture, and their use is believed to be limited to animal testing, with no human use allowed (at least, not in industrialized countries).

Either or both of those two factors – danger signals, and pathogen patterns – can trigger immune cells to travel to any site where they have been injected, and that is a highly useful trait, for adjuvants. Accordingly, a truly well-designed adjuvant or adjuvant combination ideally should be able to perform both of those two functions.

It also should be noted that some experts divide adjuvants into two main categories, depending on a different feature. In this approach, "delivery" adjuvants increase immune responses, by means that increase the amount of contact between antigens and immune cells, while "immuno-stimulatory" adjuvants activate immune responses system by stimulating certain types of cells to release "cytokines", which are hormone-type messenger molecules, described in more detail below.

Since the vaccines described herein are intended for "topical" administration to mucous membrane surfaces, specific attention must be focused on efforts to develop adjuvants for mucosal vaccines. Rhee 2012, "Mucosal Vaccine Adjuvants Update," and Aoshi 2017, "Modes of Action for Mucosal Vaccine Adjuvants," are two review articles which summarize and analyze efforts by numerous research teams to test various candidate agents for that specific type of use.

A number of passages in Rhee 2012 focus upon and candidly discuss the risks, dangers,

and problems that arose (and, in many cases, forced the abandonment of approaches that were sufficiently promising to justify large investments by teams of skilled scientists and managers), when researchers and/or companies tried to take apparently promising types of adjuvants beyond successful animal trials, into human clinical trials that ended badly. An episode described in Mutsch 2004 is a well-known example of that type of failure. A mucosal influenza vaccine which had performed well in animal tests, was put into human use, in Switzerland; however, in humans, it ended up causing some of the recipients to suffer from a neurologic affliction called "Bell's palsy". That condition creates a partial paralysis of the facial muscles, usually on either the left or the right side of the face (rarely on both sides). As such, it can lead to strange, unsettling, sometimes bizarre, and in some cases grotesque and genuinely frightening facial expressions, among sufferers. That mucosal vaccine had to be withdrawn from the market, as soon as those cases began to appear, and the manufacturer went out of business soon thereafter, under the weight of the liabilities it incurred from its efforts to create a better (mucosal) vaccine against influenza. That unfortunate event is well and widely known among experts, and among companies that might otherwise be tempted to launch a large and expensive research project into some other new potential mucosal vaccine.

As briefly mentioned above, one of the main reasons why very little work is being done to try to translate and expand research on mucosal vaccines, in animals, into efforts to develop and test mucosal vaccines in humans, arises from the fact that most of the adjuvants which are included in mucosal vaccine formulations that are tested in animals larger than rabbits, are highly aggressive, and toxic, and involve compounds like cholera toxin, or a toxin found in many pathogenic strains of *E. coli*, called the "E. coli heat labile toxin". Those types of aggressive and potentially toxic adjuvants are used in animals – especially larger animals, such as sheep, goats, pigs, horses, etc. – to make sure that every dose of a vaccine formulation which contains one of those adjuvants, will be *very* likely to provoke an active immune response to that vaccine, in pretty much every one of the animals which is inoculated with that vaccine formulation. Very high rates of response have a much, much better chance of generating the statistical levels of probability that will satisfy statisticians and scientists. In most areas of science, a confidence level of 95% has been arbitrarily set, to establish a "high level of confidence". If even just 1 animal out of 20 inoculated animals fails to have an immune response to some particular vaccine, that can foul up and block any effort to reach a showing that has a 95% level of confidence. Therefore, adjuvants used in animal testing of vaccines tend to aim for at least 99% success rates, among inoculated animals; and, any adjuvant which is that aggressive cannot be used safely, in humans.

Four more points must also be noted, which are peculiar to adjuvants for mucosal vaccines (and, it should be noted, none of these factors are addressed directly, in either the Rhee 2012 or the Aoshi 2017 article). All of these points help demonstrate that there are no clear and distinct boundaries, when it comes to words such as "adjuvant." All of the compounds mentioned below are regarded by some experts as "delivery adjuvants", while other experts regard them as merely excipients, instead.

The first point centers on the phrase, “muco-adherent”. This refers to certain types of specialized compounds which can cause a nasal spray (or other formulation, such as a fine powder that can be treated like a spray) to cling to mucosal surfaces, inside the nasal sinuses or elsewhere, for a longer period of time than would occur in their absence. Agents which can prolong and sustain the clinging of vaccine particles, to mucosal surfaces, are assumed to increase the likelihood that the vaccine particles will be noticed, and recognized as foreign, by the mucosal immune system. Examples include:

(i) chitosan, and certain types of cyclodextrins, phospholipids, and other “bioadhesive” compounds, as described in articles such as Davis 2003 and Zuercher 2003; and,

(ii) powders that convert into sticky gels when they become wet, such as a compound from aloe vera plants sold by DelSite Biotechnologies under the trademarks GELVAC and GELSITE (www.delsite.com).

More information on vaccines formulated as aerosols, powders, or similar forms, is in articles such as LiCalsi 1999 and Zhou 2014.

The second point relates to a different class of compounds, often called “absorption enhancers” or similar terms. These compounds act as carrier-type compounds, which can help drugs or other foreign molecules penetrate into membranes more rapidly, and/or in higher quantities. Dimethyl-sulfoxide (DMSO) offers one example, and others are known. These types of compounds are often used in ointments, creams, and other formulations that are applied directly to skin, to enable aspirin or other pain-killers to reach sore muscles, joints, etc., more rapidly. As with muco-adherents, some experts regard these as delivery adjuvants, while others regard them as mere excipients.

The third point which merits attention, concerning candidate adjuvants for mucosal vaccines, is that nasal membranes (like the outer membranes of most types of animal cells) tend to be negatively charged. Therefore, use of “cationization” (pronounced as KATT-eye-onni-ZAY-shun, i.e., using steps which will attach “cat-ions”, which are positively-charged ions) to impart a positive charge to vaccine particles, can increase the contact time between the vaccine particles, and targeted mucosal membrane surfaces, which are negatively charged.

The final point that merits mention is that different experts are likely to apply the term “adjuvant” in different ways, to any phage particles which have been assembled in ways that will make them useful as vaccines, as described herein. Some experts will regard the starting-point particles (i.e., the structures of the phages themselves, which will remain largely unchanged, as the foreign peptide sequences are attached to their surfaces) as adjuvants. However, other experts will regard the phage particles as the vaccine particles, since they will be created as fully-integrated, already-assembled particles which will emerge in exactly that form, from the host bacterial cells. According to experts in the second category, proper use of the phrase, “materials added to vaccines, in order to improve the immunological response”, means that the term “adjuvants” should only be applied to things that are added to the particles, after the particles have been created (provided that any such adjuvant must rise above the minor facilitating levels of mere excipients).

EPITOPES: ANTIGEN SEQUENCES CHOSEN FOR VACCINE RESEARCH OR USE

The term "epitope" also requires attention, since it will be encountered by anyone who reads about vaccines. The prefix "epi-" derives from a Greek root which refers to an upper or outermost layer, or surface; it appears in words such as epidermis (i.e., the outermost layer of skin, covering the "dry surfaces" of an animal body), and epicenter (which is the spot on the earth's surface located directly above the "hypocenter" of an earthquake, which is the below-ground "true" center where the rock layers began sliding against each other).

In the context of designing and testing vaccine particles, "epitope" refers to relatively small segments or sequences of amino acids (usually about 10 to 12 residues long) which have been chosen for research, or actual usage, in vaccine candidates, because they are in exposed and accessible locations on the surface of a pathogen, so that if antibodies are created which will bind to those epitopes, those same antibodies also will be able to bind to the pathogens that those epitopes were derived from; or, stated in terms which approach the topic from a different angle, "epitopes" are sometimes defined as the smallest portion, of some antigen sequence, which can trigger and drive an immune response that will create antibodies which will bind to that antigen (or, alternately, killer T cells which will attack any cell which displays that antigen, on its surfaces).

The property of being positioned in exposed and accessible locations, on the surfaces of pathogens or vaccine particles, is crucially important, for antigens and epitopes. One of the factors which guided the evolution and development of the immune system, over hundreds of millions of years, was the simple fact that, if antibodies formed which would bind to protein sequences that are hidden and inaccessible inside a protein molecule (or, inside a pathogenic virus or cell), then those antibodies could not be truly effective, against that pathogen, for the simple reason that they would not be able to reach, recognize, and bind to, those hidden portions of proteins. By contrast, antibodies which bind to protein sequences that are openly exposed, on easily-accessible outer surfaces of bacteria or viruses, have far better chances of binding to those particles, and triggering useful immune responses against those pathogens.

Accordingly, most epitopes are relatively small segments (or sequences) of amino acids which are exposed, and readily accessible, on the surface of a protein; and, in most cases, the protein itself also must be exposed and accessible, on the surface of a pathogen (or, in some cases, a cancerous or other diseased cell). Therefore, in any antibody-triggering vaccine which does not contain killed or attenuated pathogens, in intact form – and which, instead, was created by genetic engineering methods – the design and arrangement of those vaccine particles must somehow position a selected epitope sequence which has been chosen as "the antigen sequence", on the surfaces of the vaccine particles, in exposed locations. Indeed, when discussing an antigen that has already been selected for use in a vaccine, the words "antigen" and "epitope" can be used interchangeably, unless the context of a specific point being made indicates otherwise, since the epitope is simply that portion of an antigen which was chosen for use, as "the antigen", in that vaccine. Therefore, careful selection of epitope sequences is crucial, in designing and

creating vaccine particles which will trigger the formation of antibodies which will bind to both:

(i) the selected epitope sequence, on the surfaces of the vaccine particles; and,

(ii) the corresponding protein segments on exposed surfaces of the pathogens (or cancerous or other diseased cells) which the vaccine is designed to protect against.

In much the same way that theories, and experiments, must both be used skillfully in good research – where theories can help researchers figure out where and how to look for interesting and important results and advances, when designing new tests and experiments, but where the theories must often be adjusted and refined, to accommodate whatever was found out by actual testing – computer modeling, and actual testing, must both be used, in determining the best candidate epitope sequences, for testing in the new types of vaccines disclosed herein.

With regard to computer modeling of proteins on the surfaces of pathogens, two statements can be made:

1. The complete amino acid sequences of nearly all of the important surface proteins, on nearly all historically important pathogens, have already been analyzed, and determined, and can be found in various databases; and,

2. The technology that can be used to analyze the exact DNA or RNA sequence of any new mutant form, of any important pathogen, has become remarkably sophisticated, fast, and efficient.

In other words, this is a problem that has already been solved, or which can be solved, for any new mutant strain of any type of pathogen, quickly and easily, using already-known technology. A simple internet search for "DNA sequence machine" will quickly generate hits showing a dozen different machines, sold by a dozen different manufacturers, but anyone interested in this field should be advised, in advance, to focus on machines sold by a company called Illumina. By using extraordinarily sophisticated chemistry, it can induce a strand of DNA to emit combinations of light pulses while it is being read, with different light pulse combinations corresponding to each of the four "bases" in DNA (abbreviated as A,T,C, and G). That technology allows multiple bases to be read, every second. Speed competitions between academic labs have driven the time required – to sequence an entire *human* genome – down to less than 8 hours; and, most of the important pathogenic microbes have genome lengths that are only very small fractions of the length of a human genome. A human genome has about 3 billion bases; by comparison, among bacterial pathogens, cholera has a genome length of about 4 million bases (i.e., only slightly more than 1/1000 the size of a human genome), and pneumonia has a genome size of about 6 million bases. Viral pathogens have even smaller genomes; influenza viruses usually have about 13.5 kilobases (which is only about 4/1,000,000 the size of a human genome), and corona viruses have genome lengths of about 30 kb (about 10/1,000,000 the size of a human genome). Therefore, as soon as a new mutant strain of some pathogen has been isolated, and delivered to a lab with a sophisticated sequencing machine (and which has been warned that an important new mutant is being sent to it, so that it can get the machine ready), its complete and entire genome can be sequenced in less than about two hours, most of which will be setup time, rather than sequencing time.

Once the DNA sequence of a surface protein, on a pathogenic microbe, has been sequenced, it can be converted into an amino acid sequence, by a computer, within less than a second, by simply using the "genetic code" (which has been fully known since the early 1960s), since each "codon" of three bases specifies a single, exact amino acid.

Then, once the complete amino acid sequence of a pathogen surface protein is known, its 3-dimensional shape can be calculated, quite accurately, by a software program called "AlphaFold". That program was developed by a group of academic labs in England, by using the Google "DeepMind" artificial intelligence system to analyze millions of known protein shapes, in ways that identified the various rules, patterns, and occasional quirks that apply and arise, each time a newly-formed protein emerges from the ribosome which made it, inside a cell. That software has been made available, for free usage by anyone who submits an amino acid sequence to its website, which is at alphafold.ebi.ac.uk. It is run by the European Bioinformatics Institute (EBI), which is part of the European Molecular Biology Laboratory (EMBL), an inter-government research organization funded by about 20 member countries in Europe.

As a historical note, anyone who is interested in how 3D protein modeling was done, before the AlphaFold program was released and began outperforming all competitors, can look into the Rosetta Commons group. That group, while it was active, encouraged any scientists who needed a 3D model of a protein, to create several distinct 3D models of that sequence (using at least several different protein modeling programs, from an assortment of more than 20 such programs which could be accessed through links maintained by the Rosetta Commons group), and to then compare those different results against each other. As this is being written, that group still has a website, at www.rosettacommons.org, and it may have some level of internal activity, but it apparently no longer replies to inquiries from outsiders.

Accordingly, computer modeling can provide accurate 3D models of any pathogenic surface protein that is of interest, in any pathogen. However, in the same way that theories and experiments must be combined with each other, in scientific research, actual testing has been able to identify very large numbers of actual and proven epitopes, as indicated by tests which determined which particular short amino sequences, on some pathogenic protein, did indeed become "bound" by so-called "neutralizing antibodies". That type of testing, which is called "epitope mapping", can be done by any of several methods, which are described in review articles such as Hamed 2023 ("State of the art in epitope mapping . . . "), which offers a good introduction and overview (a full copy is available, as a free download), and Hu 2023 ("Massively-multiplexed epitope mapping techniques for viral antigen discovery"), which focuses on certain methods which can use "high-throughput sequencing techniques", to speed up the results.

Epitope mapping, as a distinct branch of research, has been known for at least 25 years (e.g., Delisser 1999 is a review article with the simple title, "Epitope mapping," and it was published in 1999, in a journal called *Methods in Molecular Biology*). By 2003, so many research articles had appeared, describing various specific epitopes which had been identified from a wide array of different pathogens, that the National Institutes of Health created a

computerized database, to organize all of the relevant data, and make it "searchable" by anyone with a computer. The result of that work became the Immune Epitope Database (IEDB), which is funded and run by the National Institute of Allergy and Infectious Diseases (NIAID), one of the branches of the U.S. National Institutes of Health (NIH), and as of June 2024, the IEDB database contained data on more than 1,600,000 epitope peptide sequences. That database can be accessed and searched via www.iedb.org; a review article (Martini 2020, "The Immune Epitope Database and Analysis Resource Program 2003-2018: reflections and outlook") which describes how to use it can be downloaded for free at www.ncbi.nlm.nih.gov/pmc/articles/PMC6970984. It also is worth noting that the IEDB website also contains a set of "predictive tools", which can help anyone who knows the amino acid sequence, of a specific surface protein on a specific pathogen, to quickly identify several of the most promising candidates for potentially good and useful epitope sequences, which can be tested in candidate vaccine formulations; and, multi-component suite of analytical and predictive tools which can be accessed, at no cost to any researcher, via nextgen-tools.iedb.org/pipeline?tool=tc1.

As a closing comment on this topic, epitope selections are for other people, companies, and agencies to make, who have true expertise and experience in that particular field. The invention described and explained herein – i.e., mucosal vaccines which have been enhanced by adding MALT-targeting peptide sequences to their surfaces – should be regarded as analogous to delivery vehicles, comparable to a new type of truck, which is being offered, initially on a rental basis, to people who want to transport some type of cargo that they already know is suited for transport by a truck. It will be up to other people, and other companies, to choose and decide on what type of freight, or payload, or passengers, or any other suitable term, that they want to load into or onto those trucks, so that they can test that truck-and-cargo combination, for their own purposes, and using their own methods and their own trusted employees, before they decide whether they might want to enter negotiations to buy that type of truck, so that they can use it for more than just testing and evaluation.

Although any company which is just getting started must stay flexible, adaptable, and both able and willing to change its plans, in response to both developments and demands, and opportunities and openings, that arise in the real world, one of the goals and aspirations of Tetraheed Medical LLC (the startup company which owns the patent rights that are being created by this and several other, related patent applications) will be to offer to create, and then provide, at the lowest practical cost, to any qualified company or researcher, a set of MALT-targeting phage particles carrying any specific epitope sequence which that company, or that researcher, chooses and nominates. Any information about any epitope sequence which any company, person, or agency has chosen to study, or how they plan to study it, or in what type(s) of animals, or why they want to study that epitope in specific, will be kept strictly confidential, unless and until that company has seen the results of its work, and chooses to disclose that information on its own. Indeed, it will not even be necessary for an outside company or researcher to tell the phage-makers what sequence they want to have inserted into a phage cassette; instead, if they wish, they can simply send an oligonucleotide to the phage-makers, with an assurance that the

requesters have designed it to fit properly into a specified "genetic cassette" (that phrase is described below), if that "cassette" is opened up by digesting it with two specific DNA cleavage enzymes, which the submitter will specify, based on knowing the entire DNA sequence of the cassette.

Accordingly, any deeper discussion or analysis of epitope selection (or mapping) is outside the scope of this patent application. Instead, this invention discloses a new type of delivery vehicle, for delivering – to exactly the right types of immune cells, in exactly the right locations (i.e., MALT patches, which are specialized lymph nodes that are exposed, and directly accessible to vaccine particles, on the surfaces of mucous membranes) – any epitope, or any antigen sequence, which any qualified vaccine company, or any qualified vaccine researcher or immunologist, wishes to attach to the surfaces of particles which will also carry MALT-targeting sequences on their surfaces.

IMMUNE CELLS ARE ATTRACTED AND RECRUITED TO INFECTION SITES BY "CHEMOKINES" (aka CHEMO-ATTRACTANTS)

Before an effort is made to describe a complex group of signaling molecules called "*cyto*-kines", a quick description of a *different* group of signaling molecules should be completed, and then set aside. These molecules, called "*chemo*-kines", can be released by almost any type of cell, if that cell has become infected by a pathogen. The goal, purpose, and effect of any and all chemokines is simple and straight-forward: they are used to attract and recruit any nearby immune cells, to come take care of a cell which has been infected by a pathogen, and which has been commandeered and hijacked in a way which threatens to release dozens or hundreds of copies of that pathogen (which are actively reproducing inside the infected cell), unless an immune cell can reach the infected cell in time, swallow it up, and kill the pathogens.

Accordingly, chemokines are also sometimes called "chemo-attractants." That term is preferred herein, since it is much easier for non-experts to immediately understand what a "chemo-attractant" is. By contrast, if a non-expert encounters the word "chemokine," s/he is likely to have to pause, and ask, "Okay, are they talking about *chemo*kines, or *cyto*kines? And, do I remember which word means which?"

Mobile immune cells process chemo-attractant signals by using a cellular process called "chemotaxis", which is roughly equivalent to a sense of smell when a nose is not involved. Because macrophages and dendritic cells each have multiple copies of various chemotactic receptors distributed all around their surfaces, they can detect both: (i) which part of the surface (of the immune cell) the first few chemokine molecules contacted; and, (ii) which direction contains the highest concentration of chemokine molecules that an immune cell is responding to. An affected immune cell will then travel in the direction of the first contact, and then in whatever direction points toward the highest concentrations of chemokines, until it reaches an infected cell. At that point, the immune cell can engulf and surround the infected cell as rapidly as possible, to kill the infected cell and any pathogens growing inside that cell; or, in some cases, more complex signaling molecules, called *cyto*-kines, will take over, and begin initiating

different types of activation processes.

Without disclosing the details here, it is worth mentioning that a chemokine (chemo-attractant) played a crucial role in the "Second Round Screening Tests", which are described below, in the Detailed Description and Examples sections.

IMMUNE CELLS COMMUNICATE VIA "CYTOKINES"

The molecules called "hormones" and "cytokines" offer a classic example of how scientific definitions and terms can (and often must) change and evolve, over time, as more information is discovered. An early (but now obsolete) definition of "hormone" was, "a molecule which is released inside the body, and which travels through the body, and which contacts – and creates a substantial and measurable effect upon – some other cell or body part."

Later, scientists discovered a special class of hormones which were different from any of the other long-known hormones. The Greek root words "kinesis" and "kine" refer to movement and/or activation, and show up in modern words such as "kinetic", and in "kinase" enzymes, which refer to enzymes which convert inactive precursor molecules, into fully-activated molecules (often but not always by transferring a phosphate group to the precursor molecule, in a manner which will activate it).

However, problems of terminology soon arose, because the newly-discovered "hormones" were different from the types of "hormones" defined above. Among other things, the newly-discovered class of hormones could trigger and activate travel, through soft tissues in the body, by entire cells. Therefore, they were given the name "cytokines", which can be translated into, "something which can make entire cells start moving and go somewhere else, not as part of growth and maturation, but as already full-grown and mature cells."

Inevitably, disagreements arose between competing groups of experts, over whether the newly-discovered "cytokines" should be regarded as a subset of hormones, or as a separate and distinct class of signaling molecules. The experts who argued that cytokines were so different that they should never again be called "hormones" eventually won those debates; and, not surprisingly, researchers have been trying, ever since, to pin down an exact distinction and boundary line between "hormones" versus "cytokines". That effort has been rendered difficult, because – as often occurs, in biology and evolution – the two categories tend to overlap, in a few specific areas which can be called "gray zones".

During the effort to sharpen, clarify, and limit the definition of "hormone", that term gradually became defined by several limitations, and it now refers to signaling molecules:

(i) which are created and released by a known and particular organ, gland, or other fixed and stationary body part;

(ii) which travel via circulating blood (this excludes travel through the clear lymph fluids, between the cells in soft tissues), to reach some other organ or body part; and,

(iii) which, upon contacting the targeted cells or body part, create a known and predictable type of effect upon it/them.

By contrast, signaling molecules which do not meet all three of those criteria are usually

called “cytokines”. Examples of various types of signaling molecules that are outside the more exact and limited definition of “hormone” include:

(i) signaling molecules which are NOT created and then released by some discrete, known, and identifiable body part (this category includes ANY signaling molecules created by ANY mobile immune cells which travel through the body);

(ii) signaling molecules which are released into lymph fluids, rather than into circulating blood; and,

(iii) signaling molecules which are not “released” by the generating cell, and which, instead, are mounted on the surface of the generating cell, so that an immune cell which detects that molecule, on the surface of some cell, will be triggered (by that contact or exposure) to carry out some special action involving that cell (such as, for example, gobbling it up and killing it).

As a result of how those distinctions evolved, any and all *signaling molecules which are used to regulate immune cells* (and/or the immune system) are called *cytokines*, rather than hormones. No expert will ever attack anyone for using the term “cytokine” when referring to any signaling mechanism(s) of the immune system. By contrast, experts can (and often will) criticize and belittle any use (and any user) of the term “hormone” to refer to signaling molecules that affect the immune system, or immune cells.

Dozens of acronyms, as names for specific cytokines, have been created by immunologists (the molecule called [NAME DELETED] is just one of many examples), and it is impossible (and wasted effort) for anyone to try to memorize them, unless they work full time in immunology; and, part of the problem in interpreting these acronyms is that they are not used consistently. For example, a single acronym might refer to any or all of the following:

(i) a particular cytokine (i.e., the molecule which has the signaling activities and effects);

(ii) receptors which respond to that cytokine; or,

(iii) cell types which have had either that cytokine, or receptors which respond to that cytokine, mounted (in a fixed manner which endures for at least some substantial period of time) upon their surfaces.

The best reasonably short video that can be nominated and recommended herein, for readers who want to know more about how cytokines and signaling components function within an immune system – and which presents helpful illustrations of the cell types and signaling factors – can be found at www.youtube.com/watch?v=GXVLdbkRkhw. It is a lecture (with illustrated slides) by Dr. John Looney of the University of Rochester Medical School, called “T Cell Activation and Control.”

That completes this survey of the relevant prior art, which began in a number of different fields of study, but which all had to be brought together, in a complex structure and system, to create this invention. Attention will now turn to the invention itself, beginning with the Objects of the Invention, and then a short Summary, followed by short descriptions of the drawings, figures, and photographs that are part of this application, and then, by a “Detailed Description,” and Examples.

OBJECTS OF THE INVENTION

One object of this invention is to disclose, create, and provide new types of mucosal vaccines which can carry any selected antigen sequence, in large numbers per particle, and which can be administered topically to the surfaces of mucosal membranes (e.g., by a nasal spray, mouthwash, slow-release lozenge, or other formulation described herein), and which, when administered in that manner, can drive the formation and secretion of mucosal IgA antibody dimers by an inoculated human or other animal, in ways which can either: (i) supplement, accompany, and increase the levels of protection, against microbial pathogens, that can be provided by injectable vaccine formulations; or, (ii) reduce or eliminate the need for injected vaccines.

Another object of this invention is to disclose, create, and provide a new type of "platform" for simple and rapid creation of mucosal vaccines, in which any selected antigenic protein segment (or combination of antigenic sequences) from one or more pathogens can be easily and quickly incorporated into vaccine particles which have been designed and created in ways that, while carrying antigenic peptide segments in large numbers, will enable them to use a small number of "MALT-targeting" sequences to promote:

(1) rapid uptake of the vaccine particles, by M cells located in "mucosal-associated lymphoid tissue" patches (i.e., MALT patches, which can be located in naso-pharyngeal, intestinal, bronchial, genital, or other mucosal tissues);

(2) rapid "trans-cytosis" of the vaccine particles, through those M cells; and,

(3) ejection of the vaccine particles into "docking sites" behind the MALT patches, where large numbers of dendritic cells reside, and wait for such "deliveries".

Another object of this invention is to disclose new types of genetic vectors (which also can be called "genetic cassettes") which can enable the rapid and simple insertion of pathogen-derived antigen-encoding DNA sequences, into targeted insertion sites in such vectors, in a manner which will enable such vectors-with-insertions to rapidly and inexpensively create, via bacterial or other cellular fermentation, vaccine particles having both:

(i) large numbers of pathogen-derived antigenic peptide sequences, on exposed and accessible surfaces of said vaccine particles; and,

(ii) small numbers of MALT-targeting sequences which will promote uptake and processing by immune cells in surface-mounted MALT patches, as described in the prior paragraph.

Another object of this invention is to disclose new types of vaccine formulations which, when administered by a nasal spray, mouthwash, lozenge, or other topical formulation, can use a combination of: (i) MALT-targeting transport sequences, in low numbers on the particles in such formulations, and (ii) selected pathogen-derived antigenic sequences (or sequences that appear on the surfaces of cancerous or other diseased cells), in high numbers on the particles in such formulations, to drive and enable the formation and secretion of mucosal IgA antibody dimers by an inoculated human or other animal, in ways which can: (i) supplement, accompany, and increase the levels of protection, against microbial pathogens, that can be provided by injectable

vaccine formulations; (ii) reduce or eliminate the need for vaccine injections; or, (iii) enable new methods of treating various types of cancer or other diseases.

Another object of this invention is to disclose new types of mucosal vaccines that have been modified in ways which can eliminate or reduce any need to add aggressive, inflammatory, irritating, and/or potentially dangerous or toxic adjuvant compounds to the final formulations.

Another object of this invention is to disclose new types of mucosal vaccines made from nonpathogenic bacteriophage particles which have been engineered to carry, on exposed and accessible surfaces of the vaccine particles, foreign polypeptide sequences that have been shown to actively trigger uptake and processing of said vaccine particles by NALT, GALT, BALT, or other "MALT" (mucosal-associated lymphoid tissue) cells that are part of various "mucosal membrane" patches that are part of the immune system in various locations in animal bodies.

Another object of this invention is to disclose a new approach to developing and manufacturing vaccine particles, using nonpathogenic bacteriophages containing engineered genes which encode coat proteins carrying foreign polypeptide inserts that can promote delivery of the vaccine particles (including antigenic proteins carried by the vaccines) to antigen-presenting immune cells, in a rapid and efficient manner which ensures rapid and efficient processing and "antigen presentation" by the immune cells.

Another object of this invention is to disclose new types of vaccines (which can use bacteriophages, other types of viruses, or cellular microbes) that have been enhanced by incorporating into them a plurality of "MALT-targeting" transport polypeptide sequences that will trigger and drive: (i) intake into mucosal immune cells and tissues, followed by (ii) intake and processing by macrophages or dendritic cells, in ways which will lead to formation and secretion of IgA antibodies into saliva or other mucosal fluids.

Another object of this invention is to disclose new types of mucosal vaccines which can be administered as "booster doses," following a first inoculation using an injectable vaccine, to drive the formation and secretion of IgA antibodies into saliva or other mucosal fluids, in ways which can provide a recipient animal or human a "two-pronged" antibody response which uses both internal IgG antibodies (or comparable IgY antibodies in birds), and secreted IgA antibodies, to provide better immune protection, against one or more types of targeted pathogens, than can be provided by either an IgG response, or an IgA response, acting alone.

Another object of this invention is to disclose new types of vaccine particles which can be delivered more rapidly, to immature dendritic cells which have not yet committed to processing any specific type of microbial particle, than can be achieved by injectable vaccines, by incorporating, into the surfaces of such vaccine particles, polypeptide sequences which emulate "pathogen patterns," thereby enabling such vaccine particles to utilize, exploit, and take advantage of the cellular processes used by M cells in MALT tissue patches, to: (i) rapidly pull in particles carrying recognized "pathogen patterns" on their surfaces, via phagocytosis; (ii) rapidly transport such particle-carrying phagosomes through the M cells; and (iii) ejecting said particles, in "naked" form (i.e., no longer surrounded by phagosomal membranes), into cellular docking sites behind or beneath said M cells, where mobile immune cells reside while waiting to

receive pathogens that will be processed by the immune system to form antibodies.

Another object of this invention is to disclose new types of vaccines which do not require refrigeration, and which do not require injections, or needles, for administration to humans or animals.

Another object of this invention is to disclose new types of vaccines that can be manufactured very rapidly, in large quantities and at low cost, by using bacteria (or other cells that can be grown in stirred cell culture) as the host cells for a fermentation process.

These and other objects of the invention will become more apparent through the following summary, drawings, and description.

SUMMARY OF THE INVENTION

Vaccine particles and formulations are disclosed, which . . .

THE SUMMARY, LIST OF FIGURES, "DETAILED DESCRIPTION" SECTION, EXAMPLES, AND TABLES HAVE BEEN REMOVED FROM THIS NON-CONFIDENTIAL RELEASE

REFERENCES

Ackerman ME et al (2011) A robust, high throughput assay to determine the phagocytic activity of clinical antibody samples. J Immunol Methods 366:8 19.

Acres B, et al (2007) Discovery and development of therapeutic cancer vaccines. Curr Opin Drug Discov Devel 10: 185 92.

Aderem A and Underhill D (1999). Mechanisms of phagocytosis in macrophages. Ann. Rev. Immunol. 17: 593–623.

Agarwal S, Aggarwal S (2015) Mucoadhesive polymeric platform for drug delivery; a comprehensive review. Curr Drug Deliv 12:139-56.

Aghebati-Maleki L, Bakhshinejad B, Baradaran B, Motallebnezhad M, Aghebati-Maleki A et al (2016) Phage display as a promising approach for vaccine development. J Biomed Sci 23:66.

Ainai A, Tadaki Suzuki T, Tamura S, Hasegawa H (2017) Intranasal Administration of Whole Inactivated Influenza Virus Vaccine as a Promising Influenza Vaccine Candidate. Viral Immunol. 2017 30(6):451-462.

Alexopoulou L, et al (2005) Contribution of microbial-associated molecules in innate mucosal responses. Cell Mol Life Sci. 62(12):1349-58.

Alfaleh MA, Alsaab HO, Mahmoud AB, Alkayyal AA, Jones ML, Mahler SM, Hashem AM (2020) Phage Display Derived Monoclonal Antibodies: From Bench to Bedside. Front Immunol. 11: 1986

Allahyari M, Mohit E (2016) Peptide/protein vaccine delivery system based on PLGA particles. Hum Vaccin Immunother 12:806-28.

Alloatti A, Kotsias F, Magalhaes JG, Amigorena S (2016) Dendritic cell maturation and cross-presentation: timing matters! Immunol Rev 272:97-108.

Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines. Curr Opin Immunol. 2012 Jun;24(3):310-5.

Aoshi T. Modes of Action for Mucosal Vaccine Adjuvants. Viral Immunol. 2017 Jul/Aug;30(6):463-470.

Arunachalam TS, et al. Mixed oligonucleotides for random mutagenesis: best way of making them. Org

Biomol Chem. 2012 10(24): 4641-50.

Awaad A, Nakamura M, Ishimura K (2012) Histochemical and biochemical analysis of the size-dependent nanoimmunoresponse in mouse Peyer's patches using fluorescent organosilica particles. Int J Nanomedicine 7:1423-39.

Bacon A, Makin J, Sizer PJ, Jabbal-Gill I, Hinchcliffe M, Illum L, Chatfield S, Roberts M (2000) Carbohydrate biopolymers enhance antibody responses to mucosally delivered vaccine antigens. Infect Immun. 68:5764–70.

Bahadir AO, et al (2011) Phage displayed HBV core antigen with immunogenic activity. Appl Biochem Biotechnol. 165(7-8):1437-47

Bai Y, Ye L, Tesar DB, Song H, Zhao D, Bjorkman PJ, Roopenian DC, Zhu X (2011) Intracellular neutralization of viral infection in polarized epithelial cells by neonatal Fc receptor (FcRn) mediated IgG transport. Proc Natl Acad Sci U S A. 108:18406 18411

Bakhshinejad B, Sadeghizadeh M (2014) Bacteriophages as vehicles for gene delivery into mammalian cells: prospects and problems. Expert Opin Drug Deliv 11:1561 74.

Bandell A (2011) Protective efficacy of live attenuated influenza vaccine (multivalent, Ann Arbor strain): A literature review addressing interference. Expert Rev Vaccines 10:1131 41.

Bao Q, Li X, Han G, Zhu Y, Mao C, Yang M (2018) Phage based vaccines. Adv Drug Deliv Rev. 2018 pii: S0169 409X(18)30317 X.

Barabas A, Cole C, Kovacs Z, Kovacs E, Lafreniere R (2018) The Modified Vaccination Technique. Vaccines (Basel) 7. pii: E1.

Barbas CF, Burton DR, Scott JK, Silverman GJ (2004) Phage Display: A Laboratory Manual. CSHL Press.

Bauer S (2013) Toll-like receptor 9 processing: the key event in Toll-like receptor 9 activation? Immunol Lett 149:85-7

Bazan J, Całkosiński I, Gamian A (2012) Phage display--a powerful technique for immunotherapy: 2. Vaccine delivery. Hum Vaccin Immunother 8:1829-35.

Beigel JH (2018) Polyclonal and monoclonal antibodies for the treatment of influenza. Curr Opin Infect Dis 31:527-534.

Belyakov IM, Ahlers JD, Berzofsky JA (2004) Mucosal AIDS vaccines: current status and future directions. Expert Rev Vaccines 3:S65-73.

Bernard JM, Francis MB (2014) Chemical strategies for the covalent modification of filamentous phage. Front Microbiol 5:734.

Bertram U, Bernard MC, Haensler J, Maincent P, Bodmeier R (2010) In situ gelling nasal inserts for influenza vaccine delivery. Drug Dev Ind Pharm 36:581–93.

Bethell D, Saunders D, Jongkaewwattana A, Kramyu J, Thitithayanont A, Wiboonut S et al (2013) Evaluation of in vitro cross reactivity to avian H5N1 and pandemic H1N1 2009 influenza following prime boost regimens of seasonal influenza vaccination in healthy human subjects: a randomised trial. PLoS One 8:e59674.

Bethell D, Saunders D, Jongkaewwattana A, Kramyu J, Thitithayanont A, Wiboonut S et al (2013) Evaluation of in vitro cross reactivity to avian H5N1 and pandemic H1N1 2009 influenza following prime boost regimens of seasonal influenza vaccination in healthy human subjects: a randomised trial. PLoS One 8:e59674.

Bigaeva E, Doorn Ev, Liu H, Hak E (2016) Meta-Analysis on Randomized Controlled Trials of Vaccines with QS-21 or ISCOMATRIX Adjuvant: Safety and Tolerability. PLoS One 11:e0154757.

Bitsaktsis C, Babadjanova Z, Gosselin EJ (2015) In vivo mechanisms involved in enhanced protection utilizing an Fc receptor targeted mucosal vaccine platform in a bacterial vaccine and challenge model. Infect Immun 83:77 89.

Black S et al (2011) Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. Pediatr Infect Dis J 30:1081 5.

Blaisdell BE, et al (1996) Similarities and dissimilarities of phage genomes. Proc Natl Acad Sci USA 93: 5854-5859.

Blander JM (2007) Signalling and phagocytosis in the orchestration of host defence. Cellular Microbiology

9:290

Boackle SA, Morris MA, Holers VM, Karp DR (1998) Complement opsonization is required for presentation of immune complexes by resting peripheral blood B cells. J Immunol 161:6537 43.

Bonnafous P et al (2014) Treatment of influenza virus with beta propiolactone alters viral membrane fusion. Biochim Biophys Acta 1838:355 63.

Bonnycastle LLC et al (2001) General phage methods. In: Phage Display: A Laboratory Manual. Eds: Barbas CF III, Burton DR, Scott JK, Silverman GJ. Cold Spring Harbor Laboratory Press. NY.

Bowen RL (2001) Sex hormones, amyloid protein, and Alzheimer disease. JAMA 286:790-1.

Bradley RW, Buck M, Wang B (2016) Tools and Principles for Microbial Gene Circuit Engineering. J Mol Biol. 428(5 Pt B):862-88

Brandtzaeg P (2003) Mucosal immunity. Dev Biol (Basel) 115:111-7.

Brandtzaeg P (2003) Role of mucosal immunity in influenza. Dev Biol (Basel) 115:39-48.

Brandtzaeg P (2009) Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol 70:505-15.

Brandtzaeg P (2011) Immune functions of nasopharyngeal lymphoid tissue. Adv Otorhinolaryngol 72:20-4.

Brandtzaeg P (2011) Potential of nasopharynx-associated lymphoid tissue for vaccine responses in the airways. Am J Respir Crit Care Med 183:1595-604.

Brandtzaeg P (2013) Secretory IgA: Designed for Anti-Microbial Defense. Front Immunol. 4:222.

Brandtzaeg P (2013) Secretory immunity with special reference to the oral cavity. J Oral Microbiol. 2013;5.

Brandtzaeg P, Kiyono H, Pabst R, Russell MW (2008) Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal Immunol. 1(1):31-7

Brayden DJ et al (2005) Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today 10: 1145-57.

Brumell JH, Grinstein S. (2003) Role of lipid-mediated signal transduction in bacterial internalization. Cell Microbiol. 5: 287-97.

Brune KD, Leneghan DB, Brian IJ, Ishizuka AS, Bachmann MF, Draper SJ, Biswas S, Howarth M (2016) Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization. Sci Rep. 6:19234

Bublil EM, Freund NT, Mayrose I, Penn O, Roitburd-Berman A, Rubinstein ND, Pupko T, Gershoni JM (2007) Stepwise prediction of conformational discontinuous B-cell epitopes using the Mapitope algorithm. Proteins 68(1):294-304.

Buchanan KL, Murphy JW (1998) What makes Cryptococcus neoformans a pathogen? Emerg Infect Diseases 4: 71-83.

Bukreyev A, et al (2006) Nonsegmented negative strand viruses as vaccine vectors. J Virol 80: 10293 10306.

Burrows GG. (2005) Systemic immunomodulation of autoimmune disease using MHC-derived recombinant TCR ligands. Curr Drug Targets Inflamm Allergy. 4: 185-93

Cabilly S (1999) The basic structure of filamentous phage and its use in the display of combinatorial peptide libraries. Mol Biotechnol. 12 (2): 143-8

Calvopina-Chavez DG, Gardner MA, Griffitts JS (2022) Engineering efficient termination of bacteriophage T7 RNA polymerase transcription. G3 (Bethesda) 12(6):jkac070.

CDC (2018) Information on Rapid Molecular Assays, RT PCR, and other Molecular Assays for Diagnosis of Influenza Virus Infection.

Chan, H.K. (2006) Dry powder aerosol delivery systems: current and future research directions. J Aerosol Med. 19: 21 7

Chaturvedi M, Kumar M, Pathak K (2011) A review on mucoadhesive polymer used in nasal drug delivery system. J Adv Pharm Technol Res 2:215-22.

Chen L, Ruan F, Sun Y, Chen H, Liu M, Zhou J, Qin K (2019) Establishment of sandwich ELISA for

detecting the H7 subtype influenza A virus. J Med Virol. 2019 Jan 24.

Cheng W (2016) The Density Code for the Development of a Vaccine? J Pharm Sci 105:3223 3232.

Chiarantini L, Argnani R, Zucchini S, et al (1997) Red blood cells as delivery system for recombinant HSV 1 glycoprotein B: immunogenicity and protection in mice. Vaccine 15:276 280.

Chulpanova DS, Solovyeva VV, Kitaeva KV, Dunham SP, Khaiboullina SF, Rizvanov AA (2018) Recombinant Viruses for Cancer Therapy. Biomedicines 6(4):94.

Clark JR, et al, (2004) Bacterial viruses as human vaccines? Expert Rev Vaccines. 3: 463-76.

Clements ML et al (1986) Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. J Clin Microbiol 23:66 72.

Coban C, et al (2005) Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. J Leuk Biol 78: 647-655.

Cobey S, Wilson P, Matsen FA 4th (2015) The evolution within us. Philos Trans R Soc Lond B Biol Sci 370 pii: 20140235

Condron BG et al (1991) Frameshifting in Gene 10 of Bacteriophage T7. J. Bacteriol. 173: 6998-7003.

Conner JM et al (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J Neurosci. 17: 2295-313.

Cooper et al (2004) Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. Vaccine 22: 3136-3143.

Corr SC, Gahan CC, Hill C (2008) M cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol 52:2 12.

Court DL et al (2002) Genetic engineering using homologous recombination. Annu. Rev. Genet. 36: 361–88

Cranage MP, Manoussaka M (2009) Modern mucosal vaccines, adjuvants and microbicides. Expert Rev Anti Infect Ther 7:21-3.

Dale SJ et al. Oligonucleotide-directed random mutagenesis using the phosphorothioate method. Methods Mol Biol. 1996 57:369-74

Da Silva DM, Schiller JT, Kast WM (2003) Heterologous boosting increases immunogenicity of chimeric papillomavirus virus like particle vaccines. Vaccine 21:3219 27.

Davenport BJ et al (2022) Phage-like particle vaccines are highly immunogenic and protect against pathogenic coronavirus infection and disease. NPJ Vaccines 7(1):57.

Davis SS, et al (2003) Absorption enhancers for nasal drug delivery. Clin Pharmacokinet. 42: 1107-28.

de Bernardi di Valserra M, Zanasi A, Ragusa S, Gluck R, Herzog C (2002) An open label comparison of the immunogenicity and tolerability of intranasal and intramuscular formulations of virosomal influenza vaccine in healthy adults. ClinicalTherapy 24:100 11.

de Veer M, Meeusen E (2011) New developments in vaccine research--unveiling the secret of vaccine adjuvants. Discov Med 12:195-204.

Deering RP, Kommareddy S, Ulmer JB, Brito LA, Geall AJ (2014) Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines. Expert Opin Drug Deliv 11:885-99

Del Poeta M (2004) Role of phagocytosis in the virulence of Cryptococcus neoformans. Eukaryotic Cell 3: 1067-1075.

Deng L, Linero F, Saelens X (2016) Production and Purification of Recombinant Filamentous Bacteriophages Displaying Immunogenic Heterologous Epitopes. Methods Mol Biol 1404:483-95.

des Rieux A et al (2006) Nanoparticles as potential oral delivery systems. J Control Release. 116: 1-27.

Desbat B et al (2011) Effect of the beta propiolactone treatment on the adsorption and fusion of influenza A/Brisbane/59/2007 and A/New Caledonia/20/1999 virus H1N1 on a dimyristoylphosphatidylcholine/ganglioside GM3 mixed phospholipids monolayer at the air water interface. Langmuir 27:13675 83.

Desheva Y, Sychev I, Smolonogina T, Rekstin A, Ilyushina N, Lugovtsev V, Samsonova A, Go A, Lerner A (2018) Anti neuraminidase antibodies against pandemic A/H1N1 influenza viruses in healthy and influenza infected individuals. PLoS One 13:e0196771.

Desjardins M et al (2005) Phagocytosis: the convoluted way from nutrition to adaptive immunity. Immunol Rev 207: 158-165.

Elhay M, et al.(2007) Suppression of behavioural and physiological oestrus in the mare by vaccination against GnRH. Aust Vet J. 85: 39-45

Eliasson DG, Helgeby A, Schön K, Nygren C, El-Bakkouri K, Fiers W, Saelens X, Lövgren KB, Nyström I, Lycke NY (2011) A novel non-toxic combined CTA1-DD and ISCOMS adjuvant vector for effective mucosal immunization against influenza virus. Vaccine 29:3951-61.

Ellebedy AH (2018) Immunizing the Immune: Can We Overcome Influenza's Most Formidable Challenge? Vaccines (Basel) 6. pii: E68.

Ellebedy AH, Ahmed R (2012) Re engaging cross reactive memory B cells: the influenza puzzle. Front Immunol 3:53.

Enshell-Seijffers D, Smelyanski L, Gershoni JM (2001) The rational design of a 'type 88' genetically stable peptide display vector in the filamentous bacteriophage fd. Nucleic Acids Res. 29(10):E50-0

Eriksson AM, Schoen KM, Lycke NY (2004) The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues. J Immunol 173:3310-9.

Eriksson K, et al (2002) Recent advances in mucosal vaccines and adjuvants. Curr Opin Immunol. 14: 666-72.

Ermak TH, Giannasca PJ. (1998) Microparticle targeting to M cells. Adv Drug Deliv Rev. 34: 261-283.

Estrada A, Laarveld B, Li B, Redmond MJ (1995) Induction of systemic and mucosal immune responses following immunization with somatostatin-avidin complexes incorporated into ISCOMS. Immunol Invest 24:819-28.

FDA (2007) Guidance to industry: Clinical data needed to support the licensure of seasonal inactivated influenza vaccines. www.fda.gov/downloads/BiologicsBloodVaccines/

Fiers W, De Filette M, El Bakkouri K, Schepens B, Roose K, Schotsaert M, Birkett A, Saelens X (2009) M2e based universal influenza A vaccine. Vaccine 27:6280 3.

Fiorino F, Pettini E, Pozzi G, Medaglini D, Ciabattini A (2013) Prime boost strategies in mucosal immunization affect local IgA production and the type of th response. Front Immunol 4:128.

Firdaus FZ, Skwarczynski M, Toth I (2022) Developments in Vaccine Adjuvants. Methods Mol Biol. 2412:145-178.

Fournillier A, Frelin L, Jacquier E, Ahlon G, Brass A, Gerossier E et al (2013) A heterologous prime/boost vaccination strategy enhances the immunogenicity of therapeutic vaccines for hepatitis C virus J Infect Dis 208:1008 19.

Freeman SA, Grinstein S (2014) Phagocytosis: receptors, signal integration, and the cytoskeleton. Immunol Rev 262:193-215.

Frenkel D, et al (2002) Filamentous phage as vector-mediated antibody delivery to the brain. Proc Natl Acad Sci U S A. 99: 5675-9.

Frenkel D, et al (2004) Generation of anti-beta-amyloid antibodies via phage display technology. Vaccine. 22: 2505-8.

Fujihashi K, Koga T, van Ginkel FW, Hagiwara Y, McGhee JR (2002) A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine 20:2431-8.

Fukuizumi T, Inoue H, Anzai Y, Tsujisawa T, Uchiyama C (1995) Sheep red blood cell instillation at palatine tonsil effectively induces specific IgA class antibody in saliva in rabbits. Microbiol Immunol 39:351-9.

Fukuizumi T, Inoue H, Tsujisawa T, Uchiyama C (1997) Tonsillar application of killed Streptococcus mutans induces specific antibodies in rabbit saliva and blood plasma without inducing a cross reacting antibody to human cardiac muscle. Infect Immun 65:4558 63.

Fukuizumi T, Inoue H, Tsujisawa T, Uchiyama C (2000) Streptococcus sobrinus antigens that react to salivary antibodies induced by tonsillar application of formalin killed S. sobrinus in rabbits. Infect Immun 68:725 31.

Fukuizumi T, Inoue H, Tsujisawa T, Uchiyama C (2000) Streptococcus sobrinus antigens that react to

salivary antibodies induced by tonsillar application of formalin killed S. sobrinus in rabbits. Infect Immun 68:725 31.

Fukuyama Y, Okada K, Yamaguchi M, Kiyono H, Mori K, Yuki Y (2015) Nasal Administration of Cholera Toxin as a Mucosal Adjuvant Damages the Olfactory System in Mice. PLoS One 10:e0139368.

Futami J et al (2001) Preparation of potent cytotoxic ribonucleases by cationization: Enhanced cellular uptake and decreased interaction with ribonuclease inhibitor by chemical modification of carboxyl groups. Biochem 40: 7518-7524.

Gagnon E et al (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages, Cell 110: 119-131.

Galen JE, Curtiss R 3rd (2014) The delicate balance in genetically engineering live vaccines. Vaccine 32:4376-85.

Galimberti D, Ghezzi L, Scarpini E (2013) Immunotherapy against amyloid pathology in Alzheimer's disease. J Neurol Sci 333:50-4.

Gao J, Liu Z, Huang M, Li X, Wang Z (2011) T7 phage displaying latent membrane protein 1 of Epstein-Barr virus elicits humoral and cellular immune responses in rats. Acta Virol. 55(2):117-21

Gaubin M et al (2003) Processing of filamentous bacteriophage virions in antigen-presenting cells targets both HLA class I and class II peptide loading compartments. DNA Cell Biol. 22: 11-8.

Gelinas DS et al (2004) Immunotherapy for Alzheimer's disease. Proc Natl Acad Sci USA 101 Suppl2: 14657-14662.

Georgieva Y, Konthur Z (2011) Design and screening of M13 phage display cDNA libraries. Molecules 16:1667-81.

Ghebrehewet S, MacPherson P, Ho A (2016) Influenza. BMJ 355:i6258.

Gizurarson S (2012) Anatomical and Histological Factors Affecting Intranasal Drug and Vaccine Delivery. Curr Drug Deliv 9:566–582.

Gray M, Botelho RJ (2017) Phagocytosis: Hungry, Hungry Cells. Methods Mol Biol 1519:1-16.

GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm091990.pdf

Guo C, Manjili MH, Subjeck JR, Sarkar D, Fisher PB, Wang XY (2013) Therapeutic cancer vaccines: past, present, and future. Adv Cancer Res 119:421-75.

Gursel M, et al (2002) CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells. Eur J Immunol 32: 2617-2622.

Guy B, et al (2005) New adjuvants for parenteral and mucosal vaccines. Therapie. 60: 235-241

Han Y-, Liang A-, Han L, Guo A-, Jiang X-, Yang L- (2014) Efficacy and safety of an oral somatostatin DNA vaccine without antibiotic resistance gene in promoting growth of piglets. Scand J Immunol 79:244-50.

Heesters BA, Chatterjee P, Kim YA, Gonzalez SF, Kuligowski MP, Kirchhausen T, Carroll MC (2013) Endocytosis and recycling of immune complexes by follicular dendritic cells enhances B cell antigen binding and activation. Immunity 38:1164 75.

Heidl S, Ellinger I, Niederberger V, Waltl EE, Fuchs R (2016) Localization of the human neonatal Fc receptor (FcRn) in human nasal epithelium. Protoplasma 253:1557 1564.

Helander HF, Fändriks L. (2014) Surface area of the digestive tract - revisited. Scand J Gastroenterol 49:681-9.

Hemmi H, et al (2005) TLR signalling and the function of dendritic cells. Chem Immunol Allergy. 86: 120-35.

Henry C, Palm AE, Krammer F, Wilson PC (2018) From Original Antigenic Sin to the Universal Influenza Virus Vaccine. Trends Immunol 39:70 79.

Henry KA, Arbabi-Ghahroudi M, Scott JK (2015) Beyond phage display: non-traditional applications of the filamentous bacteriophage as a vaccine carrier, therapeutic biologic, and bioconjugation scaffold. Front Microbiol 6:755.

Hess KL, Jewell CM (2019) Phage display as a tool for vaccine and immunotherapy development. Bioeng Transl Med. 5(1):e10142.

Higgins LM, Lambkin I, Donnelly G, Byrne D, Wilson C, Dee J, Smith M, O'Mahony DJ (2004) In vivo phage display to identify M cell-targeting ligands. Pharm Res 21:695-705.

Hioe CE, Kumar R, Upadhyay C, Jan M, Fox A, Itri V, Peachman KK, Rao M, Liu L, Lo NC, Tuen M, Jiang X, Kong XP, Zolla-Pazner S (2018) The use of immune complex vaccines to enhance antibody responses against neutralizing epitopes on HIV 1 envelope gp120. Vaccine 28:352 60.

Hioe CE, Kumar R, Upadhyay C, Jan M, Fox A, Itri V, Peachman KK, Rao M, Liu L, Lo NC, Tuen M, Jiang X, Kong XP, Zolla-Pazner S (2018) The use of immune complex vaccines to enhance antibody responses against neutralizing epitopes on HIV 1 envelope gp120. Vaccine 28:352 60.

Hirata K, Kawabuchi M (2002) Myelin phagocytosis by macrophages and non-macrophages during Wallerian degeneration. Microsc Res Tech 57: 541-581.

Ho PP, et al (2003) An immunomodulatory CpG oligonucleotide for the treatment of autoimmunity via the innate and adaptive immune systems. J Immunol 171: 4920-4926.

Hodyra K, Dąbrowska K (2015) Molecular and chemical engineering of bacteriophages for potential medical applications. Arch Immunol Ther Exp (Warsz) 63:117-27.

Hoft DF, Lottenbach KR, Blazevic A, Turan A, Blevins TP, Pacatte TP, Yu Y, Mitchell MC, Hoft SG, Belshe RB (2017) Comparisons of the Humoral and Cellular Immune Responses Induced by Live Attenuated Influenza Vaccine and Inactivated Influenza Vaccine in Adults. Clin Vaccine Immunol. 24. pii: e00414-16.

Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11:S45-53.

Hosseinidoust Z (2017) Phage Mediated Gene Therapy. Curr Gene Ther 17:120 126.

Houde M, et al (2003) Phagosomes are competent organelles for antigen cross-presentation. Nature 425: 402-6.

Hunter RL (2002) Overview of vaccine adjuvants: present and future. Vaccine 20 Suppl 3:S7-12.

Hwang HS, Puth S, Tan W, Verma V, Jeong K, Lee SE, Rhee JH (2018) More robust gut immune responses induced by combining intranasal and sublingual routes for prime boost immunization. Hum Vaccin Immunother. 14:2194 2202.

Iho S, Maeyama J, Suzuki F (2015) CpG oligodeoxynucleotides as mucosal adjuvants. Hum Vaccin Immunother 11:755-60.

Inoue H, Fukuizumi T, Tsujisawa T, Uchiyama C (1999) Simultaneous induction of specific immunoglobulin A producing cells in major and minor salivary glands after tonsillar application of antigen in rabbits. Oral Microbiol Immunol 14:21 6.

Ishibashi Y, Arai T (1990) Roles of the complement receptor type 1 (CR1) and type 3 (CR3) on phagocytosis and subsequent phagosome-lysosome fusion in Salmonella-infected murine macrophages. FEMS Microbiol Immunol 2:89-96.

Ito M, Yamayoshi S, Murakami K, Saito K, Motojima A, Nakaishi K, Kawaoka Y (2019) Characterization of Mouse Monoclonal Antibodies Against the HA of A(H7N9) Influenza Virus. Viruses 11. pii: E149.

Izurieta P, Uy Aragon MJ, Dram M, Vaughn DW (2016) Assessment of Prime boost Vaccination Using an AS03B adjuvanted Influenza A (H5N1) Vaccine: A Randomized Trial in Children of Three to Less Than Eighteen Years of Age. Pediatr Infect Dis J 35:e35 47.

Jackson ML, Chung JR, Jackson LA, Phillips CH, Benoit J, Monto AS, Martin ET, Belongia EA, McLean HQ, Gaglani M, Murthy K, Zimmerman R, Nowalk MP, Fry AM, Flannery B (2017) Influenza Vaccine Effectiveness in the United States during the 2015-2016 Season. N Engl J Med 377:534-543.

Jafari N, Abediankenari S (2015) Phage Particles as Vaccine Delivery Vehicles: Concepts, Applications and Prospects. Asian Pac J Cancer Prev 16:8019 29.

Jang YH, Kim JY, Byun YH, Son A, Lee JY, Lee YJ, Chang J, Seong BL (2018) Pan Influenza A Protection by Prime Boost Vaccination with Cold Adapted Live Attenuated Influenza Vaccine in a Mouse Model. Front Immunol 9:116.

Jepson CD, March JB. (2004) Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. Vaccine. 22: 2413-9.

Jiang W, Wang S, Chen H, Ren H, Huang X, Wang G, Chen Z, Chen L, Chen Z, Zhou P (2017) A Bivalent

Heterologous DNA Virus Like Particle Prime Boost Vaccine Elicits Broad Protection against both Group 1 and 2 Influenza A Viruses. J Virol 91. pii: e02052 16.

Jilek S, et al (2005) DNA-loaded biodegradable microparticles as vaccine delivery systems and their interaction with dendritic cells. Adv Drug Del Rev 57: 377-390.

Jung T, Kamm W, Breitenback A, Hungerer KD Hundt E, Kissel T (2001) Tetanus toxoid loaded nanoparticles from sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide): evaluation of antibody response after oral and nasal application in mice. Pharm Res 18:352-60.

Jurchenko C et al (2014) Intergrin generated forces lead to streptavidin biotin unbinding in cellular adhesions. Biophysical J 106:1436 1446.

Jutras I, Desjardins M (2005) Phagocytosis: At the Crossroads of Innate and Adaptive Immunity. Annu Rev Cell Devel Biol 21: 511-527.

Kammona O, Bourganis V, Karamanidou T, Kiparissides C (2017) Recent developments in nanocarrier aided mucosal vaccination. Nanomedicine (Lond) 12:1057 1074

Karimi M, Mirshekari H, Moosavi Basri SM, Bahrami S, Moghoofei M, Hamblin MR (2016) Bacteriophages and phage inspired nanocarriers for targeted delivery of therapeutic cargos. Adv Drug Deliv Rev 106:45 62.

Karlin S, et al (1994) Why is CpG suppressed in the genomes of virtually all small eukaryotic viruses but not in those of large eukaryotic viruses? J Virol 68: 2889-2897.

Kensil CR, Patel U, Lennick M, Marciani D (1991) Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex. J Immunol 146:431-7

Kim SH, Jang YS (2014) Antigen targeting to M cells for enhancing the efficacy of mucosal vaccines. Exp Mol Med 46:e85.

Kirchhoff OP et al. Random mutagenesis by using mixtures of dNTP and dITP in PCR. Methods Mol Biol. 1996 57:351-6

Kiyono H, et al (2004) NALT- versus Peyer's-patch-mediated mucosal immunity. Nat Rev Immunol. 4: 699-710.

Klinman DM et al (2003) Hierarchical recognition of CpG motifs exepressed by immunostimulatory oligodeoxynucleotides. Clin Exp Immunol 133: 227-32.

Klinman DM, et al (2004) Use of CpG oligodeoxynucleotides as immune adjuvants. Immunol Rev. 199: 201-16.

Knutson KL, et al (2005) Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol Immunother. 54: 721-8

Koch S, et al (2004) Treatment and prevention of enterococcal infections--alternative and experimental approaches. Expert Opin Biol Ther. 4: 1519-31.

Koping-Hoggard M, Sanchez A, Alonso MJ (2005) Nanoparticles as carriers for nasal delivery. Expert Rev Vaccines 4:185-96.

Koppolu B, Zaharoff DA (2013) The effect of antigen encapsulation in chitosan particles on uptake, activation and presentation by antigen presenting cells. Biomaterials 34:2359-69.

Koppolu BP, Smith SG, Ravindranathan S, Jayanthi S, Suresh Kumar TK, Zaharoff DA (2014) Controlling chitosan-based encapsulation for protein and vaccine delivery. Biomaterials 35:4382-9.

Kozlowski PA, et al (2002) Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. J Immunol. 169: 566-74.

Krammer F (2016) Novel universal influenza virus vaccine approaches Curr Opin Virol. 17:95 103.

Krammer F, Palese P (2013) Influenza virus hemagglutinin stalk based antibodies and vaccines. Curr Opin Virol 3:521 30.

Krieg AM, et al (1998) Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. Proc Natl Acad Sci USA 95: 12631-12636.

Krieg AM. (2002) CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol. 20: 709-60.

Kuipers OP. Random mutagenesis by using mixtures of dNTP and dITP in PCR. Methods Mol Biol. 1996 57:351-6

Kunert R, Reinhart D (2016) Advances in recombinant antibody manufacturing. Appl Microbiol Biotechnol 100:3451 61.

Kuper CF, et al (1992) The role of nasopharyngeal lymphoid tissue. Immunol Today. 13: 219-24.

Lang D (2001) Safety Evaluation of Toxin Adjuvants Delivered Intranasally. http://www.niaid.nih.gov/dmid/enteric/intranasal.htm

Lawson MR, Berger JM (2019) Tuning the sequence specificity of a transcription terminator. Curr Genet. 65(3):729-733

Lee BK, Yun Y, Park K (2016) PLA micro- and nano-particles. Adv Drug Deliv Rev Del 107:176-191.

Leifer CA, et al (2003) Heterogeneity in the human response to immunostimulatory CpG oligodeoxynucleotides. J Immunother 26: 313-9.

Levine MM. (2003) Can needle-free administration of vaccines become the norm in global immunization? Nat Med. 9: 99-103.

Levine MZ, Holiday C, Liu F, Jefferson S, Gillis E, Bellamy AR, Tumpey T, Katz JM (2017) Cross Reactive Antibody Responses to Novel H5Nx Influenza Viruses Following Homologous and Heterologous Prime Boost Vaccination with a Prepandemic Stockpiled A(H5N1) Vaccine in Humans.J Infect Dis 216(suppl_4):S555 S559.

LiCalsi C, Christensen T, Bennett JV, Phillips E, Witham C. (1999) Dry powder inhalation as a potential delivery method for vaccines. Vaccine.17:1796-803.

Lin SC, Liu WC, Lin YF, Huang YH, Liu JH, Wu SC (2013) Heterologous prime boost immunization regimens using adenovirus vector and virus like particles induce broadly neutralizing antibodies against H5N1 avian influenza viruses. Biotechnol J 8:1315 22.

Lindesmith LC, Mallory ML, Debbink K, Donaldson EF, Brewer Jensen PD, Swann EW, Sheahan TP, Graham RL, Beltramello M, Corti D, Lanzavecchia A, Baric RS (2018) Conformational Occlusion of Blockade Antibody Epitopes, a Novel Mechanism of GII.4 Human Norovirus Immune Evasion. mSphere 3. pii: e00518 17.

Liu MA (2011) DNA vaccines: an historical perspective and view to the future. Immunol Rev 239:62-84.

Liu XB, Liang AX, Feng XG, Guo AZ, Ke CY, Zhang SJ, Yang LG (2011) Oral and intranasal administration of somatostatin DNA vaccine mediated by attenuated Salmonella enterica serovar Typhimurium to promote growth of piglets. Animal 5:1231-6

Liu YC, et al (2000) Cultivation of recombinant Escherichia coli to achieve high cell density with a high level of penicillin G acylase activity. Proc Natl Sci Counc Repub China B 24: 156-60.

Lo DD (2013) Mucosal vaccine delivery: is M cell-targeted delivery effective in the mucosal lumen? Expert Opin Drug Deliv 10:157-61.

Luhrmann A, Haas A (2001) A method to purify bacteria-containing phagosomes from infected macrophages. Meth Cell Sci 22: 329-341.

Lycke N (2012) Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 12:592-605.

Lycke N. (2004) From toxin to adjuvant: the rational design of a vaccine adjuvant vector, CTA1-DD/ISCOM. Cell Microbiol. 6: 23-32.

Lycke NY, Bemark M (2012) The role of Peyer's patches in synchronizing gut IgA responses. Front Immunol 3:329.

Macarron R, Banks MN, Bojanic D, Burns DJ, Cirovic DA, Garyantes T, Green DV, Hertzberg RP, Janzen WP, Paslay JW, Schopfer U, Sittampalam GS (2011) Impact of high-throughput screening in biomedical research. Nat Rev Drug Discov 10:188-95.

Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P (2008) The immune geography of IgA induction and function. Mucosal Immunol 1:11-22.

Magnani M, Chiarantini L, Vittoria E, Mancini U, Rossi L, Fazi A (1992) Red blood cells as an antigen delivery system. Biotechnol Appl Biochem16:188 194.

Mairhofer J, Wittwer A, Cserjan-Puschmann M, Striedner G (2015) Preventing T7 RNA polymerase read-through transcription-A synthetic termination signal capable of improving bioprocess stability. ACS Synth Biol. 4(3):265-73

Makrides SC (1996) Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev. 60(3):512-38.

Malik B, Goyal AK, Markandeywar TS, Rath G, Zakir F, Vyas SP (2012) Microfold-cell targeted surface engineered polymeric nanoparticles for oral immunization. J Drug Target 20:76–84.

Man AL, Prieto-Garcia ME, Nicoletti C (2004) Improving M cell mediated transport across mucosal barriers: do certain bacteria hold the keys? Immunology 113:15-22.

Mann JF, Acevedo R, Campo JD, Pérez O, Ferro VA (2009) Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert Rev Vaccines 8:103-12.

Marasini N, Skwarczynski M, Toth I (2017) Intranasal delivery of nanoparticle-based vaccines. Ther Deliv 8:151-167.

Marć MA, Domínguez-Álvarez E, Gamazo C (2015) Nucleic acid vaccination strategies against infectious diseases. Expert Opin Drug Deliv 12:1851-65.

Matrosovich M et al (2006) New low viscosity overlay medium for viral plaque assays. Virol J 3:63.

Mazanec MB, Coudret CL, Fletcher DR (1995) Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. J Virol 69:1339-43.

McCarthy MW, Kockler DR (2004) Trivalent intranasal influenza vaccine, live. Ann Pharmacother. 38:2086-93.

McGreal EP, et al (2004) Divergent roles for C-type lectins expressed by cells of the innate immune system. Mol Immunol. 41: 1109-21

McGuire MJ, Li S, Brown KC (2009) Biopanning of phage displayed peptide libraries for the isolation of cell-specific ligands Methods Mol Biol. 504:291-321.

McKenzie A, Watt M, Gittleson C (2010) ISCOMATRIX vaccines: Safety in human clinical studies. Hum Vaccin 6. pii: 10754

Meeusen EN, Scheerlinck JP, Wattegedera S, Entrican G. (2004) Advances in mucosal vaccination. Anim Health Res Rev 5:209-17.

Mestecky J, et al (2005) Immune response versus mucosal tolerance to mucosally administered antigens. Vaccine. 23: 1800-3.

Mestecky J, Michalek SM, Moldoveanu Z, Russell MW (1997) Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. Behring Inst Mitt. 1997:33 43.

Mestecky J, Moldoveanu Z, Elson CO (2005) Immune response versus mucosal tolerance to mucosally administered antigens. Vaccine 23:1800-3

Mestecky J, Moldoveanu Z, Novak M, Compans RW (1994) Mucosal immunity and strategies for novel microbial vaccines. Acta Paediatr Jpn 36:537-44

Miedzybrodzki R, et al (2005) Bacterial viruses against viruses pathogenic for man? Virus Res. 110: 1-8.

Mohn KG et al (2016) Live attenuated influenza vaccination in children induces B cell responses in tonsils. J Infect Dis 214:722 731.

Mohn KG et al (2018) Immune responses after live attenuated influenza vaccination. Hum Vaccin Immunother 14:571 578.

Moingeon P. (2002) Strategies for designing vaccines eliciting Th1 responses in humans. J Biotechnol. 98: 189-98

Moradpour S, Sardari S (2017) Chemical aspects of nano carriers for vaccine delivery. Health Biotechnology and Biopharma 1:5 24.

Morrison CA, Williams J, Perry BN (1984) Adjuvant-free immunological manipulation of livestock. Res Vet Sci 37:108-13.

Mozdzanowska K, Feng J, Eid M, Kragol G, Cudic M, Otvos L Jr, Gerhard W (2003) Induction of influenza type A virus specific resistance by immunization of mice with a synthetic multiple antigenic peptide

vaccine that contains ectodomains of matrix protein 2. Vaccine 21:2616 26.

Murphy TV, Smith PJ, Gargiullo PM, Schwartz B. J Infect Dis. 187(8): 1309-13 (2003) The first rotavirus vaccine and intussusception: epidemiological studies and policy decisions.

Murray AM, Pearson IF, Fairbanks LD, Chalmers RA, Bain MD, Bax BE (2006) The mouse immune response to carrier erythrocyte entrapped antigens. Vaccine 24:6129 6139.

Mutsch M et al (2004) Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 350:896 903.

Muzykantov VR (2010) Drug delivery by red blood cells: vascular carriers designed by mother nature. Expert Opin Drug Deliv 7:403 27.

Muzykantov VR, Murciano JC (1996) Attachment of antibody to biotinylated red blood cells: immunored blood cells display high affinity to immobilized antigen and normal biodistribution in rats. Biotechnol Appl Biochem 24:41 45.

Nachbagauer R, Krammer F, Albrecht RA (2018) A Live Attenuated Prime, Inactivated Boost Vaccination Strategy with Chimeric Hemagglutinin Based Universal Influenza Virus Vaccines Provides Protection in Ferrets: A Confirmatory Study. Vaccines (Basel) 6. pii: E47.

Nagamoto T, Hattori Y, Takayama K, Maitani Y (2004) Novel chitosan particles and chitosan-coated emulsions inducing immune response via intranasal vaccine delivery. Pharm 21:671-4.

Neu KE, Henry Dunand CJ, Wilson PC (2016) Heads, stalks and everything else: how can antibodies eradicate influenza as a human disease? Curr Opin Immunol 42:48 55.

Nicholson KG et al (2003) Influenza. Lancet 362:1733 45.

Nielsen CH, Fischer EM, Leslie RG (2000) The role of complement in the acquired immune response. Immunology 100:4 12.

Nielsen CH, Matthiesen SH, Lyng I, Leslie RG (1997) The role of complement receptor type 1 (CR1, CD35) in determining the cellular distribution of opsonized immune complexes between whole blood cells: kinetic analysis of the buffering capacity of erythrocytes. Immunology 90:129 37.

Nishihara T, Iwabuchi T, Nohno T (1994) A T7 promoter vector with a transcriptional terminator for stringent expression of foreign genes. Gene 145(1):145-6

O'Hagan DT (2001) Recent developments in vaccine delivery systems. Curr Drug Targets Infect Disord. 1: 273-86.

O'Hagan DT, Rappuoli R (2004) Novel approaches to vaccine delivery. Pharm Res 21:1519-30

Ogra PL, Faden H, Welliver RC (2001) Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 14:430 45.

O'Hagan DT, Rappuoli R (2004) Novel approaches to vaccine delivery. Pharm Res 21:1519-30

O'Neill LA, Golenbock D, Bowie AG (2013) The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol 13:453-60.

Owen RL (1999) Uptake and transport of intestinal macromolecules and microorganisms by M cells in Peyer's patches a personal and historical perspective. Semin Immunol 11:157 63.

Oyewumi MO, Kumar A, Cui Z (2010) Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines 9:1095-107.

Pabst R (2015) Mucosal vaccination by the intranasal route. Nose associated lymphoid tissue (NALT) Structure, function and species differences. Vaccine 33:4406 13.

Palma, M (2023) Aspects of Phage-Based Vaccines for Protein and Epitope Immunization. Vaccines (Basel) 11(2): 436.

Parmley SF, Smith GP (1988) Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73:305-18.

Pasare C, et al (2005) Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol. 560: 11-8.

Pascual M, Schifferli JA (1995) Another function of erythrocytes: transport of circulating immune complexes. Infusionsther Transfusionsmed 22:310 5.

Paules CI, Sullivan SG, Subbarao K, Fauci AS (2018) Chasing Seasonal Influenza The Need for a Universal Influenza Vaccine. N Engl J Med 378:7 9.

Pavot V, Rochereau N, Lawrence P, Girard MP, Genin C, Verrier B, Paul S (2014) Recent progress in HIV vaccines inducing mucosal immune responses. AIDS 28:1701-18.

Pawlina, W (2015). Histology a Text & Atlas (7th ed.). pp. 670–678.

Pedebos C, Pol-Fachin L, Pons R, Teixeira CV, Verli H (2014) Atomic model and micelle dynamics of QS-21 saponin. Molecules 19:3744-60.

Perrin P et al (1995) Inactivation of DNA by beta propiolactone. Biologicals, 23:207 211.

Petrova VN, Russell CA (2018) The evolution of seasonal influenza viruses. Nat Rev Microbiol 16:47-60.

Phalipon A, Cardona A, Kraehenbuhl JP, Edelman L, Sansonetti PJ, Corthésy B (2002) Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo.
Immunity 17: 107-15

Pham GH, Iglesias BV, Gosselin EJ (2014) Fc receptor targeting of immunogen as a strategy for enhanced antigen loading, vaccination, and protection using intranasally administered antigen pulsed dendritic cells. Vaccine 32:5212 20

Pini A, et al (2000) Phage display of antibody fragments. Curr Protein Pept Sci. 1: 155-69.

Porter KR, Raviprakash K (2017) DNA Vaccine Delivery and Improved Immunogenicity. Curr Issues Mol Biol 22:129-138.

Pouresmaeil M, Azizi-Dargahlou S (2023) Factors involved in heterologous expression of proteins in E. coli host. Arch Microbiol. 205(5):212.

Prisco A, De Berardinis P (2012) Filamentous bacteriophage fd as an antigen delivery system in vaccination. Int J Mol Sci 13:5179-94.

Qimron U. et al (2006) Genome wide screens for E coli genes affecting growth of T7 bacteriophage. PNAS 103: 19039–19044.

Rakonjac J et al (2011) Filamentous bacteriophage: biology, phage display and nanotechnology applications. Curr Issues Mol Biol 13:51 76.

Rakonjac J, Russel M, Khanum S, Brooke SJ, Rajič M (2017) Filamentous Phage: Structure and Biology. Adv Exp Med Biol. 1053:1-20

Ramachandra L, et al (1998) Flow analysis of MHC molecules and other membrane proteins in isolated phagosomes. J Immunol Meth 219: 53-71.

Ratzschke O, Falk K, Strominger JL (1997) Superactivation of an immune response triggered by oligomerized T cell epitopes. Proc Natl Acad Sci U S A. 94:14642 7

Reese S, Dalamani G, Kaspers B (2006) The avian lung-associated immune system: a review. Vet Res. 37(3):311-24.

Rhee JH, Lee SE, Kim SY (2012) Mucosal vaccine adjuvants update. Clin Exp Vaccine Res. 1:50-63.

Riese P, Sakthivel P, Trittel S, Guzmán CA (2014) Intranasal formulations: promising strategy to deliver vaccines. Expert Opin Drug Deliv 11:1619-34.

Rosloniec EF, et al (2002) Paradoxical roles of IFN-gamma in models of Th1-mediated autoimmunity. Arthritis Res. 4: 333-6

Ruseva MM, Heurich M (2014) Purification and characterization of human and mouse complement C3. Methods Mol Biol 1100:75 91.

Samoylov A, Cochran A, Schemera B, Kutzler M, Donovan C, Petrenko V, Bartol F, Samoylova T (2015) Humoral immune responses against gonadotropin releasing hormone elicited by immunization with phage-peptide constructs obtained via phage display. J Biotechnol 216:20-8.

Santambrogio L, Sato AK, Carven GJ, Belyanskaya SL, Strominger JL, Stern LJ (1999) Extracellular antigen processing and presentation by immature dendritic cells. Proc Natl Acad Sci U S A. 96:15056-61.

Santambrogio L, Sato AK, Fischer FR, Dorf ME, Stern LJ (1999) Abundant empty class II MHC molecules on the surface of immature dendritic cells. Proc Natl Acad Sci U S A 96:15050-55.

Sartorius R, Bettua C, D'Apice L, Caivano A, Trovato M, Russo D, Zanoni I, Granucci F, Mascolo D,

Barba P, Del Pozzo G, De Berardinis P (2011) Vaccination with filamentous bacteriophages targeting DEC-205 induces DC maturation and potent anti-tumor T-cell responses in the absence of adjuvants. Eur J Immunol 41:2573-84.

Sasai M, Yamamoto M (2013) Pathogen recognition receptors: ligands and signaling pathways by Toll-like receptors. Int Rev Immunol 32:116-33.

Schenk D, et al (2004) Current progress in beta-amyloid immunotherapy. Curr Opin Immunol. 16: 599-606.

Schoeder CT, et al (2021) Modeling Immunity with Rosetta: Methods for Antibody and Antigen Design. Biochemistry 60(11):825-846.

Schwarz TF, Horacek T, Knuf M, Damman HG, Roman F, Dramij M, Gillard P, Jilg W (2009) Single dose vaccination with AS03 adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults. Vaccine 27:6284 90.

Shah HB, Smith K, Wren JD, Webb CF, Ballard JD, Bourn RL, James JA, Lang ML (2019) Insights From Analysis of Human Antigen Specific Memory B Cell Repertoires. Front Immunol 9:3064.

Shah RR, Hassett KJ, Brito LA. Overview of Vaccine Adjuvants: Introduction, History, and Current Status. Methods Mol Biol. 2017;1494:1-13.

Shahiwala A, Vyas TK, Amiji MM (2007) Nanocarriers for systemic and mucosal vaccine delivery. Recent Pat Drug Deliv Formul 1:1 9.

She YM et al (2013) Surface modifications of influenza proteins upon virus inactivation by beta propiolactone. Proteomics 13:3537 47.

She YM et al (2013) Surface modifications of influenza proteins upon virus inactivation by beta propiolactone. Proteomics 13:3537 47.

Shephard MJ, Todd D, Adair BM, Po AL, Mackie DP, Scott E (2003) Immunogenicity of bovine parainfluenza type 3 virus proteins encapsulated in nanoparticle vaccines, following intranasal administration to mice. Res Vet Sci 74:187-90.

Shima S and Sakai H (1977) Polylysine produced by Streptomyces. Agricultural and Biological Chemistry. 41: 1807 1809.

Shirota H, et al (2004) Suppressive oligodeoxynucleotides inhibit Th1 differentiation by blocking IFN and IL-12-mediated signaling. J Immunol 173: 5002-5007.

Silva AL, Soema PC, Slütter B, Ossendorp F, Jiskoot W (2016) PLGA particulate delivery systems for subunit vaccines: Linking particle properties to immunogenicity. Hum Vaccin Immunother 12:1056-69.

Sioud M (2009) Does our current understanding of immune tolerance, autoimmunity, and immunosuppressive mechanisms facilitate the design of efficient cancer vaccines? Scand J Immunol 70:516-25.

Slütter B, Bal S, Keijzer C, Mallants R, Hagenaars N, Que I, Kaijzel E, van Eden W, Augustijns P, Löwik C, Bouwstra J, Broere F, Jiskoot W (2010) Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine 28: 6282-91.

Smeal SW, Schmitt MA, Pereira RR, Prasad A, Fisk JD (2017) Simulation of the M13 life cycle Virology 500:259-274

Smith AM (2018) Host pathogen kinetics during influenza infection and coinfection: insights from predictive modeling. Immunol Rev 285:97 112.

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228:1315 7.

Smith GP (1993) Surface display and peptide libraries. Gene 128: 1-2.

Smith GP and Fernadez A-M (2004) Effect of DNA copy number on genetic stability of phage-displayed peptides. BioTechniques 36:610-604.

Smith GP, Gingrich TR (2005) Hydroxyapatite chromatography of phage display virions. Biotechniques 39: 879-884.

Smith GP, Petrenko VA (1997) Phage Display. Chem. Rev. 97: 391 410.

Smith GP, Schultz DA, Ladbury JE (1993) A ribonuclease S peptide antagonist discovered with a bacteriophage display library. Gene 128:37 42.

Solomon B (2005) Generation of anti-beta-amyloid antibodies via phage display technology towards Alzheimer's disease vaccination. Vaccine 23:2327-30.

Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, Jacobs A, et al (2008) Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs PLoS One 3:e2257.

Srivastava A, Gowda DV, Madhunapantula SV, Shinde CG, Iyer M (2015) Mucosal vaccines: a paradigm shift in the development of mucosal adjuvants and delivery vehicles. APMIS 123:275-88.

Staquicini DI et al (2021A) Targeted Phage Display-based Pulmonary Vaccination in Mice and Non-human Primates. Med(NY). 2(3):321-342

Staquicini DI et al (2021B) Design and proof-of-concept for targeted phage-based COVID-19 vaccination strategies with a streamlined cold-free supply chain. Proc Natl Acad Sci U S A. 118(30):e2105739118

Steensma DP, Shampo MA, Kyle RA (2014) Bruce Beutler: innate immunity and Toll-like receptors. Mayo Clin Proc 89:e101.

Stevceva L, et al (2004) Mucosal HIV vaccines: where are we now? Curr HIV Res. 2: 1-10.

Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol. 189(1):113-30

Sugimura Y, Hosono M, Wada F, Yoshimura T, Maki M, Hitomi K (2006) Screening for the preferred substrate sequence of transglutaminase using a phage displayed peptide library: identification of peptide substrates for TGASE 2 and Factor XIIIA. J Biol Chem 281:17699 706.

Sulczewski FB et al (2018) Nanoparticle vaccines against viral infections. Arch Virol 163:2313 2325.

Sun HX, Xie Y, Ye YP (2009) ISCOMs and ISCOMATRIX. Vaccine 27:4388-401.

Swanson JA, Hoppe AD (2004) The coordination of signaling during Fc receptor-mediated phagocytosis. J Leukoc Biol. 76: 1093-103

Tabor S, Richardson CC (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci U S A 82(4):1074-8

Tack BD, Prahl JW (1976) Third component of human complement: purification from plasma and physicochemical characterization. Biochemistry 15:4513 21.

Tamura S, Ainai A, Suzuki T, Kurata T, Hasegawa H (2016) Intranasal Inactivated Influenza Vaccines: a Reasonable Approach to Improve the Efficacy of Influenza Vaccine? Jpn J Infect Dis. 69(3):165-79.

Tamura S, Yajima A, Hatori E, Tamura S, Asanuma H, Suzuki Y, Aizawa C, Kurata T (1997) Effects of frequent intranasal administration of adjuvant-combined influenza vaccine on the protection against virus infection. Vaccine 15:1784-90.

Tang JX, et al (1997) Opposite effects of electrostatics and steric exclusion on bundle formation by F-actin and other filamentous polyelectrolytes. Biochem 36: 12600-12607.

Terauchi Y et al (2018) IgA polymerization contributes to efficient virus neutralization on human upper respiratory mucosa after intranasal inactivated influenza vaccine administration. Hum Vaccin Immunother. 14(6):1351-1361.

Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol. 72(2):211-22.

Thomas WR, Hales BJ, Smith WA. (2005) Genetically engineered vaccines. Curr Allergy Asthma Rep. 5: 197-203.

Thompson DL (2000) Immunization against GnRH in male species (comparative aspects). Anim Reprod Sci 60-61:459-69.

Thrane S et al (2016) Bacterial superglue enables easy development of efficient virus-like particle based vaccines. J Nanobiotechnology 14:30

Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A et al (2008) Heterosubtypic neutralizing monoclonal antibodies cross protective against H5N1 and H1N1 recovered from human

IgM+ memory B cells. PLoS One 3:e3942.

Treanor JJ, Tierney EL, Zebedee SL, Lamb RA, Murphy BR (1990) Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol. 64:1375-7.

Trombetta ES, et al (2005) Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 23: 975-1028.

Tsunoda R, Nakayama M, Onozaki K, Heinen E, Cormann N, Kinet Denodl C, Kojima M (1990) Isolation and long term cultivation of human tonsil follicular dendritic cells. Virchows Arch B Cell Pathol Incl Mol Pathol 59:95 105.

Uittenbogaard JP et al (2011) Reactions of beta propiolactone with nucleobase analogues, nucleosides, and peptides: implications for the inactivation of viruses. J Biol Chem 28636198 214

Vajdy M, et al (2004) The role of adjuvants in the development of mucosal vaccines. Expert Opin Biol Ther. 5: 953-65.

Vajdy M, Srivastava I, Polo J, Donnelly J, O'Hagan D, Singh M (2004) Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunol Cell Biol 82:617-27

van Ginkel FW, et al (2000) Vaccines for mucosal immunity to combat emerging infectious diseases. Emerg Infect Dis. 6: 123-32.

van Ginkel FW, et al (2005) Enterotoxin based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract. Infect Immun 73: 6892 902.

Van Reeth K, Gracia JCM, Trus I, Sys L, Claes G, Versnaeyen H, Cox E, Krammer F, Qiu Y (2017) Heterologous prime boost vaccination with H3N2 influenza viruses of swine favors cross clade antibody responses and protection. NPJ Vaccines 2. pii: 11.

Vemula SV et al (2016) Current Approaches for Diagnosis of Influenza Virus Infections in Humans. Viruses 8:96.

Ventola CL (2009) Off label drug information: regulation, distribution, evaluation, and related controversies. P T 34:428 40.

Verheust CP et al (2010) Contained use of bacteriophages: Risk assessment and biosafety recommendations. Appl biosafety 15: 32 44.

Vieira J et al (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis … Gene 19: 259–268

Vila A, Sanchez A, Evora C, Soriano I, McCallion O, Alonso MJ, (2005) PLA-PEG particles as nasal protein carriers: the influence of particles size. Int J Pharm 292: 43-52.

Villa CH, Anselmo AC, Mitragotri S, Muzykantov V (2016) Red blood cells: Supercarriers for drugs, biologicals, and nanoparticles and inspiration for advanced delivery systems. Adv Drug Deliv Rev 106:88 103.

Villa CH, Cines DB, Siegel DL, Muzykantov V (2017) Erythrocytes as Carriers for Drug Delivery in Blood Transfusion and Beyond. Transfus Med Rev 31:26 35.

Villard S, et al (2003) Peptide decoys selected by phage display block in vitro and in vivo activity of a human anti FVIII inhibitor. Blood 102: 949 952.

Wagner E, Zatloukal K, Cotten M, Kirlappos H, Mechtler K, Curiel DT, Birnstiel ML (1992) Coupling of adenovirus to transferrin polylysine/DNA complexes greatly enhances receptor mediated gene delivery and expression of transfected genes. Proc Natl Acad Sci U S A. 89:6099 103.

Wagner H, et al (2004) Targeting split vaccines to the endosome improves vaccination. Curr Opin Biotech 15: 538-542.

Wang LF, et al (2004) Epitope identification and discovery using phage display libraries: applications in vaccine development and diagnostics. Curr Drug Targets. 5: 1-15

Wang M, Gao Z, Zhang Z, Pan L, Zhang Y (2014) Roles of M cells in infection and mucosal vaccines. Hum Vaccin Immunother 10:3544-51.

Wang S, Liu H, Zhang X, Qian F (2015) Intranasal and oral vaccination with protein-based antigens: advantages, challenges and formulation strategies. Protein Cell 6:480-503.

Wang SH, Thompson AL, Hickey AJ, Staats HF (2012) Dry powder vaccines for mucosal administration:

critical factors in manufacture and delivery. Curr Top Microbiol Immunol 354:121-56.

Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, Palese P (2010) Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. PLoS Pathog 6:e1000796.

Wang XY, Wang B, Wen YM (2019) From therapeutic antibodies to immune complex vaccines. NPJ Vaccines 4:2.

Weinberg A, Song LY, Walker R, Allende M, Fenton T et al; IMPAACT P1057 Team (2010) Anti influenza serum and mucosal antibody responses after administration of live attenuated or inactivated influenza vaccines to HIV infected children. J Acquir Immune Defic Syndr 55:189 96.

Weiss DJ, Liggitt D, Clark JG (1997) In situ histochemical detection of beta galactosidase activity in lung: assessment of X Gal reagent in distinguishing lacZ gene expression and endogenous beta galactosidase activity. Hum Gene Ther 8:1545 54.

Wewestzer K, et al (2005) Phagocytosis of O4+ axonal fragments in vitro by p75- neonatal rat olfactory ensheathing cells. Glia 49: 577-587.

Wiley JA, et al (2001) Antigen-specific CD8(+) T cells persist in the upper respiratory tract following influenza virus infection. J Immunol 167: 3293-99.

Wines BD, Powell MS, Parren PW, Barnes N, Hogarth PM (2000) The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. J Immunol 164:5313 8.

Wong P et al (2003) CD8 T cells responses to infectious pathogens. Ann Rev Immunol. 21: 29-70.

Woodrow KA, Bennett KM, Lo DD (2012) Mucosal vaccine design and delivery. Annu Rev Biomed Eng 14:17-46.

Woof JM, Kerr MA (2006) The function of immunoglobulin A in immunity. J Pathol 208:270-82.

Wrammert J, Smith K, Miller J, Langley WA, Kokko K et al (2008) Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature 453:667-71.

Wu Z, Liu K (2021) Overview of vaccine adjuvants. Med Drug Discovery 11: 100-103

Xie H, et al (2003) CpG-induced immunomodulation and intracellular bacterial killing a chicken macrophage cell line. Devel Comp Immunol 27: 823-834.

Xu H, Bao X, Lu Y, Liu Y, Deng B, Wang Y, Xu Y, Hou J (2017) Immunogenicity of T7 bacteriophage nanoparticles displaying G-H loop of foot-and-mouth disease virus Vet Microbiol. 205:46-52

Xu WZ, et al (1994) A new genetically engineered vaccine for animal growth promotion. Sci China B. 37: 1234 41

Yacoby I, Benhar I (2008) Targeted filamentous bacteriophages as therapeutic agents. Expert Opin Drug Deliv 5:321-9.

Yang W, Liu X, Wang X (2023) The immune system of chicken and its response to H9N2 avian influenza virus. Vet Q. 43(1):1-14.

Yanisch-Perron, C. et al (1985) Improved M13 phage cloning vectors and host strains. Gene 33: 103–119

Yoshino N, et al (2004) A novel adjuvant for mucosal immunity to HIV 1 gp120 in nonhuman primates. J Immunol 173: 6850 7

Yue H, Li Y, Yang M, Mao C (2022) T7 Phage as an Emerging Nanobiomaterial with Genetically Tunable Target Specificity. Adv Sci (Weinh). 9(4):e2103645

Yuki Y, et al (2003) New generation of mucosal adjuvants for the induction of protective immunity. Rev Med Virol. 13: 293-310.

Zacher AN 3rd, Stock CA, Golden JW 2nd, Smith GP (1980) A new filamentous phage cloning vector: fd-tet. Gene. 9 (1-2):127-40

Zakeri B et al (2012) Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. Proc Natl Acad Sci USA. 109(12):E690–E697.

Zarnitsyna VI, Ellebedy AH, Davis C, Jacob J, Ahmed R, Antia R (2015) Masking of antigenic epitopes by antibodies shapes the humoral immune response to influenza. Philos Trans R Soc Lond B Biol Sci 370. pii:

20140248.

Zarnitsyna VI, Lavine J, Ellebedy A, Ahmed R, Antia R (2016) Multi epitope Models Explain How Pre existing Antibodies Affect the Generation of Broadly Protective Responses to Influenza. PLoS Pathog 12:e1005692.

Zerbs S, Giuliani S, Collart F (2014) Small-scale expression of proteins in E. coli. Methods Enzymol. 536:117-31.

Zhou QT, Tang P, Leung SS, Chan JG, Chan HK (2014) Emerging inhalation aerosol devices and strategies: where are we headed? Adv Drug Deliv Rev 75:3-17.

Zhu D, Tuo W (2016) QS-21: A Potent Vaccine Adjuvant. Nat Prod Chem Res 3 pii: e113

Zuercher AW (2003) Upper respiratory tract immunity. Viral Immunol 16:279-89.