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CHAPTER

6

A review of hypersensitivity methods to detect immune responses to SARS-CoV-2

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Abbreviations

ACE2	angiotensin-converting enzyme 2
ADCC	antibody mediated cellular cytotoxicity
AGEP	acute generalized exanthematous pustulosis
BCR	B-cell receptor
BAT	basophil activation test
CMV	cytomegalovirus
COVID-19	coronavirus disease 2019
CoviDCELL®	register name of Spike DTH
DTH	delayed-type hypersensitivity
DRESS	drug reaction with eosinophilia and systemic symptoms
E	envelope structural protein of SARS-CoV-2
EIA	enzyme immunoassays
FDE	fixed drug exanthema
G&C	Gell & Coombs
HLA	human leukocyte antigen
HCoV	human coronavirus
IDT	intradermal tests
IGRA	interferon gamma release assays

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LMW	low molecular weight
M	membrane structural protein of SARS-CoV-2
MERS-CoV	Middle Eastern respiratory syndrome coronavirus
N	nucleocapsid structural protein of SARS-CoV-2
NK	natural killer cells
NSAIDs	non-steroidal anti-inflammatory drugs
Nsps	non-structural proteins of SARS-CoV-2
POC	the point-of-care
PPD	purified protein derivative
RBD	envelope spike protein receptor binding domain (RBD) of SARS-CoV-2
S	spike structural protein of SARS-CoV-2
SARS-HCoV	Severe Acute Respiratory Syndrome human coronavirus
SARS-CoV-2	severe acute respiratory syndrome-coronavirus-2
SCID	severe combined immunodeficiency
SJS	Stevens–Johnson syndrome
SLE	systemic lupus erythematosus
SMX	sulphamethoxazole
SPT	skin prick test
TCR	T cell receptor
TEN	toxic epidermal necrolysis
TH2-IgE	type II immunity
TST	tuberculin skin test (Mantoux)
WHO	World Health Organization

1 Historical perspective

The expression "delayed type hypersensitivity" was introduced in the immunological vocabulary by the British immunologists Philip Gell and Robert Coombs (Gell & Coombs, 1963), in their seminal classification that categorized hypersensitivity diseases of immune origin into four classes (Table 1), according to their particular effector mechanisms. In its original meaning, the word "hypersensitivity" denoted the status of a mammalian organism immunized against a microbial pathogen and its ability to react against it after a new exposure to the same agent. But the enormous size of the repertoire expressed by the adaptive immune system enables the recognition of an astonishing number of antigenic structures, that extend well beyond the substances present in infectious (microbial) and non-infectious (ectoparasites, chemical and toxic compounds) agents, and include molecules present in the host tissues (tumour cells, autoantigens). Although the immune system machinery is under a strict control regime, subtle alterations in its functioning can result in exaggerated reactions that may cause damage to the tissues of the host. As the 1960s progressed, the dysfunction of the immune system was increasingly recognized as a pathogenic mechanism, counteracting the previous appreciation, firmly rooted in the first few decades of the 20th century, of its beneficial effects in the prevention and resolution of infectious diseases. In the context in which G&C gave birth to their celebrated classification, the word hypersensitivity was employed to describe exclusively the

Table 1 Classification of hypersensitivity reactions.

		Type II			Type IV			
	Type I	**a**	**b**	**Type III**	**a**	**b**	**c**	**d**
Latency	Immediate (seconds to minutes)	Min to hours	Min to hours	Up to 12 h	12 h to days	12 h to days	12 h to days	12 h to days
Immune reactant	IgE	IgG/IgM	IgG/IgM	IgG/IgM	CD4+ TH1 cells	CD4+ TH2 cells	CD8+ T cells	CD4+ TH17 cells
Antigens Foreign/self	Eukaryotic antigens enzymes, toxins, venoms, xenobiotics Drugs acting as haptens	Cell membrane molecules Extracellular matrix Drugs (hapt)	Cell membrane molecules	Soluble antigens Bacterial antigens Viral particles Antibody pharmaceuticals	Intracellular bacteria, virus	Parasitic worms Medications (haptens, p-i)	Virus Medications Chemicals (haptens, p-i)	Medications (haptens, p-i)
Effector mechanism	Mast cell degranulation IgE mediated Histamine, leukotrienes	C′ deposition Phagocytosis ADCC PMN influx	Interference with cell function	Immunocomplex C′ deposition, PMN influx.	Macrophage activation Granuloma formation	Eosinophilic inflammation	Cytotoxic	Neutrophilic infiltration
Beneficial Reactions	Parasitic expulsion, toxin removal: increased peristalsis, mucous secretion, edema, diarrhoea	Extracellular bacteria lysis.		Clearance bacterial antigens and viral particles	Control Mycobacterial infection	Granuloma eggs from helminths	Virus removal	Enhance phagocytosis bacteria/virus
Detrimental reactions	Allergic diseases, anaphylaxis	MBT/Rh/HA Autoimmune hypothyroidis, Good Pasture Pemphigus Rheumatic fever	Myasthenia gravis, Graves disease, Chronic idiopathic urticaria	Serum sickness Arthus reaction SLE, reactive arthritis, polyarteritis nodosa, allergic alveolitis, PSGN	Insulitis	DRESS syndrome	SJS/TEN Contact dermatitis	Pustular psoriasis DAGEP

ADCC, antibody dependent cellular cytotoxicity; DAGEP, drug induced acute generalized exanthematous pustulosis; DRESS: drug reaction with eosinophilia and systemic symptoms; HA, hemolytic anaemia; MBT, mismatched blood transfusion; PSGN, post-streptococcal glomerulonephritis; Rh, rhesus incompatibility; SJS/TEN, Stevens-Johnson syndrome/toxic epidermal necrolysis; SLE, systemic lupus erythematosus.

harmful reactions that occurred during immune responses. In an effort to avoid its ambiguous meaning, G&C describe these pathogenic responses as "allergic reactions producing tissue damage".

The G&C classification divides hypersensitivity reactions into four pathophysiological categories. The first three types describe reactions conveyed by antibodies and are considered "immediate" because its manifestations occur within the first 24 h after the initial triggering event, whereas the fourth type described hypersensitivity reactions accomplished by the T cell arm of the immune system and is considered "delayed" because the reactions are not seen until 24–48 h. The great amount of advances in our understanding of the functioning of the immune system since the year of G&C report, have led to a re-interpretation of their classification. While types I and III have remained unchanged since G&C devised their classification of hypersensitivity reactions, types II and IV have been subclassified in two and four subtypes, respectively, and a fifth type of hypersensitivity reaction has been proposed to accommodate sarcoid diseases. Despite these advances, we think that the simple G&C classification has withstood the test of time reasonably well and is still widely used to describe the pathologies resulting from unwanted reactions of the immune responses.

But it must be considered that the system devised by G&C to categorize deleterious reactions induced by rather innocuous substances can be easily applied to categorize beneficial reactions used by the immune system that allows the host to get rid of microbial invaders. In this regard, *in vivo* tests based on the G&C principles are currently used not only to diagnose patients who have experienced hypersensitivity reactions to certain substances but also to investigate the immune status of individuals affected by a particular microbial infection. Both the skin tests to demonstrate immune reaction to certain substances (contact dermatitis) and the tuberculin reaction are both diagnostic procedures contemplated as G&C Type IV reactions that reveal either a pathological hypersensitivity event or a normal immune response to a past microbial infection. Well before the discovery of T cell recirculation and the existence of skin resident memory cells, Richard Wagner wrote, in his emotive homage to the figure of Clemens von Pirquet, *"Out of the darkness of inner parts of the body and submerged tissues, the pathological processes and reactions were projected onto the surface and moved into bright light"* (Wagner, 1964). Today's immunologists take advantage of this projection and have in their hands a simple and affordable method to investigate the immune reaction to a wide variety of microbial and non-microbial challenges.

It is important to take into account that some clinical symptoms may overlap among the different classes of hypersensitivity and that many small molecular weight drugs can cause all types of hypersensitivity reactions, mostly involving type I or type IV hypersensitivity reactions. Adverse reactions to drugs (medications) are particularly important in clinical practice (Edwards & Aronson, 2000) and were defined by the World Health Organization (WHO) in 1972 as "a response to a drug which is noxious and unintended, and which occurs at doses normally used in humans for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function".

In this chapter, the general characteristics of each of the types of hypersensitivity described in Gell and Coombs classification will be summarized and the immune mechanisms involved in its four categories (Table 1), with special reference to type IV reactions including their application to the study of Covid-19 responses.

2 General overview, classification and description of hypersensitivity reactions

2.1 Type I hypersensitivity-immediate/IgE mediated

In the first category of the G&C classification, the hypersensitivity reaction was due to cell-bound antibodies of the IgE class, and is distinguished by a time lag of seconds to minutes between exposure to the allergenic substance and the onset of symptoms. The idea that this reaction was a consequence of the activity of the immune system was originally advanced by Von Pirquet and Shick (1903) and later von Pirquet coined the word allergy to describe the adverse effect induced by the reaction (Von Pirquet, 1906). The observation that a serum from an allergic subject could transfer immediate hypersensitivity to the skin of a non-allergic subject (the Prausnitz–Küstner test) (Prausnitz & Kustner, 1921) triggered the search for the molecule responsible for the hypersensitivity reaction. Despite the efforts of many laboratories, the nature of this factor, soon named by Coca and Grove (1923) as "atopic reagin", remained elusive for the next four decades. Late in the 1960s, when all other antibody classes had been discovered and G&C had devised their hypersensitivity classification, the "reaginic antibody" was independently identified by two laboratories. The new immunoglobulin, the 5th antibody class and the rarest of the serum immunoglobulins, was finally designated immunoglobulin E at the WHO meeting in Lausanne in 1968 (Bennich et al., 1968). Antibodies of the IgE class have been only found in mammals.

Although best known as the mediator of type I hypersensitivity to many inanimate substances, IgE antibodies were first considered a fundamental component of the immunity against multicellular parasites, particularly nematodes (Jarret & Miller, 1982). However, it was soon recognized that IgE was also produced in response to other non-infectious and innocuous environmental substances that do not have in common any chemical characteristics that define them as allergens (Galli, Tsai, & Piliponsky, 2008). This apparent innocuity was challenged by the fact that allergenic molecules include xenobiotics (poison ivy), enzymes (proteases from pollen and dust mites, phospholipase A2 from Hymenoptera venoms), toxins (ricin), venoms (from biting arthropods, cnidaria, reptiles) and irritants (diesel exhaust particles) (Palm, Rosenstein, & Medzhitov, 2012). This wide range of biological activities can have potentially harmful effects on the host and in this regard, the allergic reaction can be considered to be a rapid immune response that protects mammals against acute toxicity (Profect, 1991).

Allergens can enter the body by inhalation, ingestion, injection or by skin or mucosal contact. It has been observed that the repeated penetration of an antigen transmucosally and at very low doses is a particularly efficient way of inducing IgE

responses. IgE synthesis is a prototypical thymus-dependent response requiring the help of the TH2 subset of T lymphocytes (type II immunity) (Del Prete, 1992). In the inductive phase of the immune recognition, allergenic molecules that enter the body through the epithelial barriers are taken up and processed by dendritic/Langerhans cells. Allergenic proteins contain T cell epitopes that are selectively presented to CD4+ T helper cells associated to Class II HLA molecules. In the absence of danger signals of microbial origin, cytokines produced by damaged epithelial cells (Il-33) (Zhao & Hu, 2010) or mechanically injured dendritic cells (TSPL) polarize T cells to acquire a T helper type 2 (TH2) phenotype (Liu, 2006; Oyoshi, Larson, Ziegler, & Geha, 2010). B cells recognize allergenic molecules through their B-cell receptor (BCR) and are triggered by the TH2 cytokines Il-4 and Il-13 to undergo class–switch recombination to IgE (and IgG4)-producing cells (De Vries, Punnonen, Cocks, de Waal Malefyt, & Aversa, 1993). The encounter between TH2 and the B cells occurs both in lymphoid germinal centres and in local mucosal sites (respiratory mucosa) (Takhar et al., 2007).

Once released into the circulation, most of the produced IgE binds to the high-affinity receptor FcεRI on the surface of mast cells and basophiles (Metzger, Kinet, Blank, Miller, & Ra, 1989). Once bound, IgE acts as a specific antigen (allergen) receptor on the surface of those cells. Re-exposure to the same allergen initiates a process of intracellular signalling after cross linking of cytophilic specific IgE, followed by cell degranulation and rapid release of preformed (histamine, tryptase/chymase) or newly synthesized lipid mediators (prostaglandin D2, leukotriene C4), that mediate an aggressive early inflammatory reaction within 10–15 min after exposure to the allergen (Tharp, 1990). A late phase reaction, characterized by further oedema and recruitment of inflammatory cells, occurs several hours after exposure and is conveyed by bioactive cytokines (Il-1, 4, 5, 13, TNF-a, GM-CSF) produced by mast cells/basophiles within 4–6 h after allergen exposure (Dispenza, 2019). The ensuing symptoms depend on the site of allergen exposure, and can vary from a local reaction (skin rash, urticaria, eczema, edema and mucus secretion, rhinitis, angioedema, bronchospasm, diarrhoea, increased intestinal peristalsis) to a systemic response (anaphylaxis) in case of oral ingestion or intravenous administration (medications, stinging insect venoms) of the allergenic substance.

The consideration of the type II immunity (TH2-IgE) as an old evolutionary system to provide protection against helminthic parasites, led to the consideration that type I hypersensitivity reactions may be mistargeted responses against innocuous, non-noxious substances (hygiene hypotheses) (Strachan, 1989). More recently, Palm et al. (2012) proposed a different interpretation of the allergic reactions, arguing that IgE antibodies play a key role in the recognition of noxious environmental substances and that the reactions triggered by IgE provide a mostly beneficial function to the host, although they can become harmful when excessive. From an evolutionary perspective, it is reasonable that untoward biological activities conveyed by environmental agents provoke a protective immune reaction. The effector arm of type II immunity is ideally suited to cope with those unwanted activities, promoting both expulsion of parasites (increase of peristaltic movements) and the elimination of potentially toxic substances

(mucus secretion, sneezing, itching, coughing, tear production, vomiting, diarrhoea, vasodilatation, appearance of exudative fluids, dilution by edema). From an evolutionary perspective, the quick and sensitive response of type II immunity would provide mammals with a singular and adversive mechanism to detect and avoid unfavourable environments.

Some of the mechanisms responsible for hypersensitivity reactions to drugs and other low molecular weight compounds are special cases of type I hypersensitivity, and may be considered hapten-driven events. The reactivity of those molecules depends on their ability to react with proteins, producing an hapten-carrier conjugate that can elicit an immune response (Landsteiner, 1945). Haptens are small molecules that are not immunogenic by themselves but become immunogenic after covalent conjugation to a macromolecule, usually a protein. The attached hapten and their surrounding carrier amino acids create a new antigenic determinant that is recognized as non self by the immune system.

Beta-lactam antibiotics, sulphanilamides, quinolones, iodinated radiocontrast media, muscle relaxants are all drugs that are able to bind covalently to proteins and induce IgE-mediated anaphylaxis or hypersensitivity reactions (Pichler, 2019). Adverse reactions to β-lactam antibiotics (penicillin, cephalosporin) is an example of drug-induced type I hypersensitivity (Parker, 1981). The β-lactam ring is opened after nucleophilic attack by free amino groups (lysine ε-amino groups) of proteins, and the exposed carbonyl moiety forms amide bonds, creating a new penicilloic antigenic determinant for antibodies (Weltzien & Padovan, 1998). Other drugs (sulphamethoxozole, SMX) act as pro-haptens, and can bind to self-proteins only after being metabolized (nitroso metabolite, SMX-NO) (Naisbitt et al., 1999). Considering the metabolic transformation of some drugs and the production of protein adducts, the existence of these drug allergies can be considered as a particular case of a hypersensitivity reaction to xenobiotics (Li & Uetrecht, 2010).

Skin tests are the accepted standard methods to investigate Type I, IgE mediated reactions. The most common test to reveal specific IgE sensitization is the skin prick test (Pepys, 1975). After 10–15 min of reaction, the presence of a raised wheal with erythema at the site of the allergen puncture of 3 mm or greater in diameter indicates the presence of specific IgE antibodies. Although prick tests correlate well with clinical findings, they do not have a high level of sensitivity. Thus, when a prick test to a particular allergen is negative but allergy is still suspected, an intradermal test (subcutaneous injection) should be used instead. Although intradermal injections may be unpleasant for the patient and the reaction may be too strong, the test permits the use of a greater amount of allergen and is more sensitive than the prick test.

Skin tests may not be suitable when there is a high risk of triggering a severe reaction or the patient has signs of eczema or psoriasis. In these cases, or when the patient is under a medication that can interfere with the test, the determination of IgE specific to a particular allergen in a serum sample is an alternative method to study potentially sensitized subjects. Derived from the old radio-allergo-sorbent-test (Wüthrich & Kopper, 1975), the enzyme immunoassays (EIA) are widely used in clinical practice, but their predictive value and sensitivity are less than traditional

skin tests. Skin tests and measurements of specific IgE antibodies in serum give complementary information for the diagnosis of allergic diseases.

When the skin test or the specific IgE immunoassays are not conclusive, the basophil activation test (BAT), that measures either histamine release (Ostergaard, Ebbensen, Nolte, & Skov, 1990) or CD63 upregulation (González-Muñoz, Villota, & Moneo, 2008) following stimulation of blood basophiles with allergen *in vitro*, emerged as a new diagnostic tool. The test tries to reproduce *in vitro* the allergic reaction in patients sensitized to particular allergens. Both EIA and BAT studies should be used in patients in whom skin challenge can cause reactions of unpredictable severity.

2.2 Type II hypersensitivity-IIa/IIb antibody mediated

Antibodies mediate the killing of extracellular pathogens *via* different mechanisms, designed to increase the phagocytic capacity of defensive cells or to induce the lysis of the invading organism. The same mechanisms are responsible for tissue injury when antibodies of the IgG or IgM class bind to antigens present on cell membranes or in the extracellular matrix, resulting in cellular damage.

These reactions in which free antibodies induce tissue damage are contemplated as Type II hypersensitivity in G&C classification. The antigens recognized in type II reactions can either be endogenous (self-antigens) or exogenous (foreign antigens lodged onto a host, such as drugs or transfused blood components). In the case of self-antigens, the mechanism of immune tolerance is breached and self-reactive antibodies are produced that attach to endogenous molecules in the tissue of the host. Symptoms of type II reactions to exogenous antigens appear after minutes to hours, and the damage is limited to the cells or tissue where the reactions take place. Type II reactions can be divided into two subtypes: type IIa and type II b.

Type IIa refers to reactions characterized by destruction of haematopoietic and non-haematopoietic cells. Antibody bound to antigens on the cellular membrane of the target cells can induce their death by three different mechanisms: ingestion by phagocyte cells, antibody mediated cellular cytotoxicity (ADCC) by natural killer cells (NK) or activation of the complement system. When the target of the autoantibodies are components of extracellular material (basal membrane, adhesion molecules), the deposited antibodies may induce tissue necrosis by disrupting the cell matrix, impairing cellular adhesion or inducing complement dependent inflammatory reactions.

Phagocytosis is enhanced when IgG antibody-coating target cells (opsonisation) bind to FcγRI/IIA receptors present on cells such as macrophages and neutrophiles (McKenzie & Schreiber, 1998). Cytotoxicity is mainly due to NK cells, that recognize the Fc region of cell-bound antibodies through their FcγRIIIA receptor (Trinchieri & Valiante, 1993), which promotes the release of preformed perforin and granzymes resulting in apoptotic death of the target cells (Peters et al., 1991). The complement system is activated by IgM or IgG antibodies bound to the membrane of target cells, resulting in cell lysis after the assembly of the membrane attack

complex (C5b-C9). However, this lytic mechanism may not be very efficient, due to the phenomenon of homologous restriction, whereby cells are protected from lysis by autologous complement by a self-recognition mechanism that inhibits the late phases of complement attack, composed of the cell membrane proteins protectin (CD59), cofactor protein MCP (CD46) and decay-accelerating factor DAF (CD55) (Gorter et al., 1996). Nevertheless, the first steps of complement fixation results in the deposition and covalent binding of iC3b to the cell membrane of blood cells, which renders these cells susceptible to phagocytosis by specialized macrophages in the liver and spleen that express the iC3b complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Takizawa, Tsuji, & Nagasawa, 1996). This particular way of cell destruction links complement fixation to phagocytosis, and enhances the deleterious effects (anaemia, neutropenia or thrombocytopenia) of type IIa hypersensitivity reactions. Moreover, complement fixation on target cells results in the local production of anaphylotoxins (C3a, C5a) that recruit polymorphonuclear leukocytes (Forema, Glovsky, Warner, Horvath, & Ward, 1996) and amplify tissue injury through the release of hydrolytic enzymes after their autolysis.

Type IIa hypersensitivity is typified by the reactions of preformed antibodies in mismatched blood transfusion (Davenport & Mintz, 2007) and in the haemolytic disease of the newborn (Rhesus incompatibility) (Murray & Roberts, 2007), which lead to alloimmune destruction of red blood cells. The different types of autoimmune destruction of blood cells (autoimmune haemolytic anaemia, ANCA-dependent neutropenias, idiopathic thrombocytopenic purpura) and organ-specific autoimmune diseases (autoimmune hypothyroidisms, anti-GBM nephritis and Goodpasture syndrome, pemphigus vulgaris) are also examples of type IIa reactions in which antibodies directed against self-antigens promote the destruction of cells and tissues (Kumar, Abbas, & Aster, 2021). A special case of type IIa hypersensitivity is rheumatic fever, in which epitope similarity between streptococcal antigens and myocardial or brain antigens may explain the presence of reacting antibodies that contribute to pathologies affecting these organs (Cunningham et al., 1989; Guilherme, Kalil, & Cunningham, 2006).

Although drug hypersensitivity reactions are most frequently mediated by IgE (Type I) or T cells (type IV), they can also induce anaemia, neutropenia, thrombocytopenia and hepatic dysfunction, all hallmarks of IgG mediated, type IIa hypersensitivity reactions. To become immunogenic, drugs act as haptens, requiring conjugation to a cell surface protein on blood cells or other carrier proteins. Antibiotics (penicillins, cephalosporins), antihypertensive drugs (alpha methyldopa), thiazides, quinidine and other drugs can induce haemolytic anaemia and thrombocytopenic purpura through this mechanism (Kaufman et al., 1993; Petz, 1993).

In type IIb hypersensitivity, autoantibodies bind to receptors on the target cells, inducing dysfunction of the affected organ. In Graves' disease, anti-thyrotropin receptor antibodies act as agonists and stimulate the thyroid gland to produce excessive amounts of thyroid hormone (hyperthyroidism) (Chen et al., 2003). In some cases of chronic idiopathic urticaria, FcεRI on mast cells are recognized by auto IgG antibodies, which causes the degranulation of the target cells and the onset of urticaria

(Niimi et al., 1996). In myasthenia gravis, autoantibodies against the acetylcholine receptor (Schönbeck, Chrestel, & Hohlfeld, 1990) present on the membrane of striated muscle cells induce internalization and reduce the efficiency of neuromuscular signal transduction.

Many common laboratory techniques (immunoassays, direct and indirect antiglobulin tests, fluorescence) are used to diagnose the pathologies associated with type II hypersensitivity reactions. Skin tests are not reliable to diagnose drug induced Type II reactions and the drug provocation tests are high-risk methods that are not widely used. *In vitro* tests to determine NK cell activity associated to type II reactions (Viel et al., 2018) are difficult to do and can only be performed in specialized laboratories.

2.3 Type III hypersensitivity-immune complex-mediated

The first historical description of a type III reaction was reported by Maurice Arthus in 1903 (Arthus, 1903). Arthus observed that when rabbits were given repeated subcutaneous injections of horse serum—a non-toxic material—during a period of time, a local reaction (erythema, edema, induration) occurred a few hours after the fourth injection (Arthus reaction). The local reaction gets worse after subsequent injections, becoming purulent and with signs of haemorrhagic necrosis. In 1905, von Pirquet and Schick extended the Arthus reaction to the blood, as they observed that repeated intravenous injections with a protective anti-diphtheria horse antiserum caused systemic complications in children that they called "serum sickness" and attributed it to "the reaction of antigen ("toxins") and antibody ("anti-toxins")" (Von Pirquet & Schick, 1905). Symptoms developed in 1 or 2 weeks after exposure to the antigen.

Type III and type II hypersensitivity reactions are similar in that antibodies of the IgG or IgM classes are implicated, but in type III reactions the antigenic molecules are soluble and not cell bound as happens in type II hypersensitivity. In type III, the antigen-antibody encounter occurs in the blood, forming circulating immune complexes which can occasionally deposit in the endothelium of blood vessels or migrate out of plasma and deposit in host tissues. Antigens can be foreign proteins either of microbial origin or pharmaceutical products dispensed in the course of medical therapies or endogenous proteins (autoimmunity). Signs of type III reactions typically occur several hours after antigen infusion in pre-sensitized individuals.

The pathogenicity of an immune complex depends on the antigen–antibody ratio. In either antibody or antigen excess, the complex is soluble, and can be easily removed by phagocytic cells or eliminated in excreted urine. Precipitating complexes occur under certain conditions (mild antigen excess) and these complexes deposit in vascular endothelium, glomerular basement membrane, synovial lining and alveolar membranes of the lung.

Antigen–antibody complexes trigger the classical complement pathway. This process leads to covalent binding of C3 to the Ig component of the immune complex and further conversion to iC3b by complement regulators (Vivanco, Muñoz, Vidarte, & Pastor, 1999). The complement activation by the antigen-antibody

complex is a double-edged sword. On the one hand, the iC3b deposited in immunocomplexes facilitates solubilisation (Whaley & Ahmed, 1989) or removal through interaction with CR1 receptors on phagocytic cells in the liver (Katyal, Sivasankar, & Das, 2001). Although it might seem counter-intuitive, another beneficial effect of C3b deposition is the disaggregation of immune complexes in smaller entities (Miller & Nussenzweig, 1975) that can be more easily engulfed by phagocytic cells (Petersen, Baatrup, Jepsen, & Svehag, 1985). An important component of humoral anti-viral responses might be the reactions initiated by complement deposition on antigen–antibody complexes, that can contribute to the elimination of antibody coated circulating viral particles (Rajan, 2003). In this context, the C3b is said to have "neutralized" the virus.

But on the other hand, anaphylatoxins (C3a, C5a) liberated during the early phase of complement fixation attract and activate polymorphonuclear leucocytes (Mayadas, Tsokos, & Tsuboi, 2009) which release mediators that cause inflammatory damage to the tissues. Anaphylatoxins can also activate local mast cells, inducing their degranulation and the release of mediators that increase vasodilatation and vasopermeability. Depending on the site of deposition, symptoms of vasculitis (of endothelial cells of blood vessels), purpuric rash (dermis), arthritis (joints) or glomerulonephritis (renal glomeruli) can develop. In the case of inhalational entry, hypersensitivity pneumonitis (allergic alveolitis) can ensue. The "farmer's lung" disease (Campbell, 1932) is a potentially dangerous hypersensitivity pneumonitis that has attained considerable importance in respiratory medicine, and afflicts agricultural workers exposed to organic material such as dust of improperly dried grains or spores of fungus that grow in certain crops.

Hypersensitivity III reactions are implicated in a number of autoimmune, infectious or drug-induced diseases. Systemic lupus erythematosus (SLE), a chronic, autoimmune inflammatory disorder of connective tissue, is a prototypic type III hypersensitivity disease, in which antibodies against nuclear components react with released chromatin from apoptotic debris forming immune complexes. In the more severe forms of SLE, these immune complexes can deposit in different organs, leading to a wide variety of abnormalities including nephritis, arthritis, cutaneous rash (small vessels) and mesenteric vasculitis or mononeuritis (medium to large size vessels) (Aranow, Diamond, & Mackay, 2008).

Infectious diseases like hepatitis B, bacterial endocarditis and yersiniosis display a continuous source of antigens to form circulating immune complexes, causing polyarteritis nodosa, poststreptococcal glomerulonephritis and reactive arthritis, respectively (Kumar et al., 2021).

At the current time, serum sickness is associated with medications containing heterologous proteins (snake antivenom immunoglobulins, anti-thymocyte globulin, protein vaccines, thrombolytic therapies, chimeric monoclonal antibodies) or with insect stings. The local injection of the antigen may cause a necrotizing skin lesion (Arthus reaction). Repeated transfusions containing residual amounts of plasma or infusion of normal plasma in IgA deficient patients may induce anaphylactoid reactions suggestive of type III hypersensitivity. Despite the wide use of medications

containing potential inducers of type III reactions, the annual rate of serum sickness incidence is low (Rixe & Tavarez, 2021).

2.4 Type IV hypersensitivity-IVa/IVb/IVc/IVd-T cell mediated

Type IV hypersensitivity reactions are delayed responses (DTH) that involve T lymphocytes as the major effector cells. The nature of the delayed skin reactions was first revealed by Karl Landsteiner, who proved that the reaction was mediated by the cellular and not the antibody arm of the immune response (Landsteiner & Chase, 1942). After the intradermal injection of antigen in a sensitized individual, a cellular infiltration producing erythema, swelling and induration occurs at the site of the lesion 24–48 h later. Antigens engulfed by phagocytic cells are presented to local T cells, which become activated and orchestrate the influx of other cell types that amplify tissue injury through the release of cytokines or lysosomal enzymes (Poulter, Seymour, Duke, Janossy, & Panayi, 1982).

Delayed hypersensitivity reactions were first described in 1890 by Robert Koch, during his failed attempts to induce protective reactions against infections with tuberculous bacilli (Koch, 1890). After intradermal injection of a filtrate of heat-killed bacilli, Koch described for the first time a delayed inflammatory response in individuals who had been exposed to the tubercule bacillus. Despite the failure to induce a protective response, the tuberculin reaction (aka as the PPD skin test) soon became an important diagnostic test for tuberculosis. Methods of local application of tuberculin were promptly developed by Maurice Mantoux (Mantoux, 1908) and Claude von Pirquet (Von Pirquet, 1909), who coined the expression "tuberculin reaction". Von Pirquet used a method of cutaneous scratch to apply tuberculin, a method that was less reproducible than the intradermal injection employed by Mantoux. Decades later, Florence Seibert obtained a purified protein derivative (PPD) by acid/salt precipitation of a tuberculin preparation (Seibert, 1934) that gave fewer non-specific reactions than tuberculin after intradermal injection, enabling the creation of a reliable test for tuberculosis.

Certain subsets of T cells are specifically designed to deal with intracellular pathogens of viral, bacterial, fungal or protozoan origin. Specifically activated T cells of the CD8+ category function to destroy infected cells, either directly (cytotoxicity) or after recruitment of other cells that participate in the immune response. Memory T cells, mostly Tαβ with an effector phenotype (Clark et al., 2006) infiltrate the skin and become tissue resident T cells that can be reactivated upon antigen encounter. In a physiological setting, these cells are part of the immune surveillance system, localized in one of the principal sites of entry of microbial pathogens (Tokura, Phadungsaksawasdi, Kurihara, Fujiyama, & Honda, 2021). Experiments conducted in the 1990s in SCID mice engrafted with human cells revealed that both CD4 and CD8 T cells were involved in delayed skin reactions to tuberculin (Tsicopoulos et al., 1998).

As it happens in other cases of hypersensitivity reactions, delayed responses can be directed against self-molecules, and this process can end up causing autoimmune

damage to the host tissues. Moreover, the adverse reactions to some drugs and chemicals also have notorious delayed type hypersensitivity manifestations, and are even more common than immediate type I allergic reactions and in some cases they can be life-threatening. Many drugs or chemicals are capable of eliciting type IV reactions, including beta-lactam antibiotics, quinolones, tetracyclines, certain NSAIDs like oxicam, X-ray contrast media, metallic ions, poison ivy, local anaesthetics, allopurinol, anticonvulsants, anticoagulants like LMW heparins, and antiviral and antifungal medications (Brandt & Bircher, 2017).

The covalent binding of drugs to self-proteins (hapten–carrier complex) can easily explain antibody-based hypersensitivity reactions, and the same mechanism can be involved in some cases of delayed type hypersensitivity reactions to medical drugs. Beta-lactam antibiotics can form covalent links with peptides bound in the groove of surface HLA molecules or react with self-proteins, that after intracellular processing, yield hapten modified peptides, giving rise to new T cell epitopes (Weltzien & Padovan, 1998). But in some cases, drugs and other chemicals that lack hapten characteristics bind noncovalently to the immune receptors involved in T cell activation, promoting the polyclonal or oligoclonal stimulation of T cells. These compounds that modified T cell reactivity are of great medical relevance. Unlike conventional type IV hypersensitivity, the induction of reactions by the pharmacological interaction of drugs with immune receptors (p-i concept, Pichler, 2008) does not require a previous drug-specific sensitization. In some cases (sulphamethoxasole), the drug alters the T cell receptor directly, initiating a signalling event that must be completed by conventional TCR-HLA interactions. In others (abacavir, carbamazepine), the drugs interact with peptide-binding pockets of certain HLA alleles inducing changes in the shape of bound self-peptides (Ramsbottom, Carr, Jones, & Rigden, 2018). This seems to be sufficient to create neoepitopes that trigger unwanted T cell responses, probably involving preactivated T cells. Abacavir, an antiretroviral agent indicated for the treatment of HIV-1 infection, binds to the peptide cleft of the allele B*5701, and induces a severe and systemic hypersensitivity reaction, precluding its use in patients expressing that allele (Mallal et al., 2002). See Fig. 1 for a more detailed description of drug-induced type IV hypersensitivity reactions.

Naïve T lymphocytes differentiate into distinct subpopulations depending on the nature of the invading antigen, the type of presenting cell and the cytokine microenvironment. Depending on the T cell subpopulation involved, type IV reactions can be further subdivided into Ia, IVb, IVc and IVd subtypes. Each of the distinct T phenotypes release certain chemokines and cytokines that preferentially recruit and activate monocytes (type IVa), eosinophils (type IVb), or neutrophils (type IVd). In type IVc reactions cytotoxic T lymphocytes participate in the direct killing of target cells.

Type IVa reactions are mediated by the TH1 lymphocyte subset. A prototypical example is the tuberculin reaction, characterized by a preferential TH1-type cytokine profile with significant increases in the numbers of IL-2 and IFN-gamma mRNA-expressing cells (Tsicopoulos et al., 1992) and activation of macrophages.

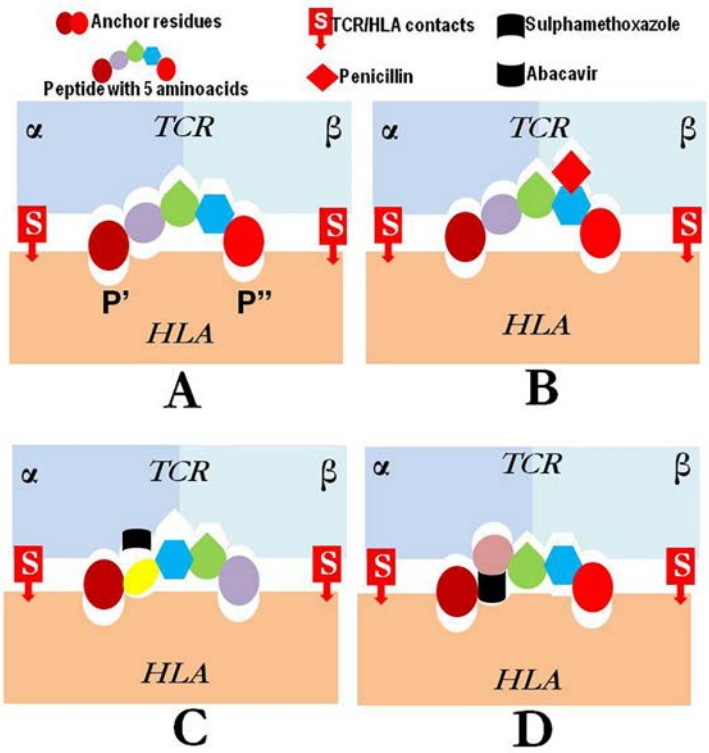

FIG. 1

Models of drug interactions with the T cell receptor/peptide/HLA complex that modify T cell reactivity. A normal event in T cell recognition is depicted in A). A processed peptide (only five aa are shown for simplicity) fits into the floor of the binding cleft of a HLA molecule. Typically, two amino acid side chains bind into particular anchor pockets (p) in the base of the groove. A TCR paratope is locked into the topside of the peptide. The trimolecular complex is further stabilized by interactions between the TCRαβ chains and the α helix domains of the HLA molecule (). In the hapten model (B), a drug/chemical (e.g., penicillin,) binds covalently to a self-protein that is processed by an APC and presented as short peptides, some of which can bear the haptenized fragment (neoepitope). If recognized by the TCR of a non-tolerized T cell, an unpredicted immune response can ensue. In (C), (*p-i* TCR concept) a drug/chemical like sulphamethoxazole () bind non-covalently to the paratope of a TCR, and alters its conformation inducing a stimulatory signal regardless of the bound peptide, that is complemented by the canonical TCR-HLA interactions. The reaction is similar to that seen in an alloimmune response. Alternatively (D), the drug (e.g., abacavir) () can bind non-covalently to the binding pocket of an HLA molecule (*p-i* HLA concept), altering the shape of the permissible peptides without the requirement of intracellular processing or allowing the attachment of a different array of peptides. The new epitopes can induce a polyclonal activation of T cells. The p-i-HLA concept has also been called “altered peptide repertoire model”.

Mycobacterium tuberculosis (a pathogen that resides inside macrophages of the host) induces a delayed type response that isolates bacteria-laden macrophages and initiates the formation of granulomas, a cluster of organized immune cells that contain/prevent the spreading of the infectious agent. Although granuloma formation is a protective mechanism, the cytokines and lytic enzymes secreted by the macrophages and other cells in the granuloma may cause extensive damage to the surrounding tissues. In most cases, granulomas, literally "small nodules", have an infectious origin (Williams & Williams, 1983), but sometimes (as in sarcoidosis, see below) the involved antigen is unknown.

One of the best known examples of autoimmune type IVa reactions is insulitis, the destruction of pancreatic insulin-producing beta cells by infiltrating T lymphocytes that occur in the early phases of insulin-dependent diabetes mellitus. It has been proposed that a previous infection with group B coxsackieviruses can precipitate the onset of insulitis (Fohlman & Friman, 1993), highlighting the association between autoimmune diseases and viral infections.

In type IVb reactions, TH2 lymphocytes produce Il-4, 5 and 13 that induce eosinophilic inflammation and allergic symptoms. Activated eosinophils can migrate and cause systemic injury (DRESS: drug reaction with eosinophilia and systemic symptoms) (Bocquet, Bagot, & Roujeau, 1996). Type IVb reactions can extend the inflammation of allergic disorders. Many medications can induce DRESS syndrome, including anti-convulsants, antibiotics, anti-inflammatory or anti-cancer drugs (Adler et al., 2017). The mechanism by which the drugs activate the T cell system involves the formation of covalent bonds with self-peptides (hapten model), creating new T cell epitopes, or inserting into the groove of HLA molecules and modifying the bound peptide (p-i concept). TH2 driven granulomatous responses to helminthic eggs can be found in the liver and intestine of patients affected with parasitic worms (Wynn, Thompson, Cheever, & Mentink-Kane, 2004).

Type IVc cytotoxic CD8 T lymphocytes kill target cells by release of the apoptosis inducers perforin/granzyme B, granulysin and Fas ligand. The better studied pathologies are Stevens-Johnson syndrome (SJS, Lyell syndrome) and toxic epidermal necrolysis (TEN). SJS and TEN are acute, potentially fatal skin reactions that are considered parts of a disease spectrum. TEN is the more severe form of the disease (Dodiuk-Gad, Chung, Valeyrie-Allanore, & Shear, 2015). The targeted cells in SJS/TEN are keratinocytes of the skin and mucous membranes that are killed by the immune attack of cytotoxic lymphocytes. Although SJS/TEN can be triggered by infections such as pneumonia, herpes virus and hepatitis, in most cases they are caused by certain medications, including antimicrobials (nevirapine), NSAIDs, drugs and anticonvulsants (Baryaliya et al., 2011).

Contact dermatitis is a classic example of a non-infectious type IV hypersensitivity reaction, an eczematous skin reaction induced by many small reactive chemicals (metal ions, latex, cosmetics, synthetic dyes, poison ivy) (Murphy, Atwater, & Mueller, 2021), that react with and modify immune receptors (p-i concept).

In Type IVd, neutrophilic infiltration causes severe inflammatory skin diseases, such as pustular psoriasis (of probable autoimmune origin) (Naik & Cowen, 2013) and acute generalized exanthematous pustulosis (associated with medications: antibiotics, antifungals, antimalarials, anti-inflammatories) (Szatkowski & Schwartz, 2015). The reaction is initiated by TH17 cells that attract neutrophiles after secreting CXCL-8 (Il-8) and Il-17 (Lochmatter, Zawodniak, & Pichler, 2009).

A special case of hypersensitivity is sarcoidosis, a systemic granulomatous disease of unknown origin. In a prototypical granulomatous-type hypersensitivity, TH1/TH2 cells orchestrate a reaction to wall off macrophages laden with undigested mycobacteria/metazoan parasites preventing pathogen spread. In the case of sarcoidosis, the nature of the offending antigen is not clear. Some authors have suggested that sarcoidosis should be considered a fifth type of hypersensitivity reaction (Rajan, 2003), that would include granulomatous responses to foreign inanimate material. Early attempts to identify the antigen in spleen extracts of patients suffering from sarcoidosis (Kveim reaction) (Chase, 1961), were not conclusive. More recently, bacterial DNA was identified in sarcoidosis lesions, suggesting that mycobacteria could be important players in the pathogenesis of sarcoidosis (Song et al., 2005). On the other hand, recent findings reveal that T cells from sarcoidosis patients recognize peptides derived from several self-molecules including vimentin and β-actin. An intriguing possibility is that, in sarcoidosis patients, molecular mimicry drives a T cell response to certain microbial antigens into an autoimmune reaction (Wahlstrom et al., 2009). In this respect, sarcoidosis could be considered a special case of type IVa reactions.

DTH skin reactions may be used to reveal previous exposures to intracellular pathogens (fungi, bacteria). Antigenic material is injected intradermally into the skin. Appearance of swelling and erythema usually >5–10 mm in 48–72 h indicates that the subject is already exposed to the antigen. Of clinical relevance are DTH skin reactions to detect past infections with *M. tuberculosis* (Mantoux test), *Mycobacterium leprae* (lepromin test) or fungi (Candida, Coccidioides). Although once common, DTH reactions, used for the purpose of detecting a past infection, are now only employed for tuberculosis screening.

Delayed type hypersensitivity reactions to chemicals are best studied by a patch test and it is widely used in predicting sensitivity to contact allergens. Patch tests are safer and more comfortable for the patient than intradermal tests.

Cell mediated immunity can be easily tested by DTH intradermal skin testing. In this respect, DTH reactions are a useful method to investigate T cell anergy in immunocompromised or immunodeficient patients. Screening requires the use of several common recall antigens. Two is the minimum number of antigens required to detect delayed hypersensitivity in 100% of normal subjects (Gordon, Krouse, Kinney, Stiehm, & Klaustermeyer, 1983). Problems of antigen availability can be overcome by using a multiple test device (Multitest CMI, Institute Mèrieux), that permits the simultaneous intradermal application of seven ubiquitous recall antigens: candida, tuberculin (PPD), tetanus, diphtheria, trichophyton, streptococcus and proteus.

3 Skin test application of hypersensitivity reactions: *In vivo* measurements of immune responses

Skin tests are diagnostic tools for identifying the mechanisms of hypersensitivity to substances. The major methods routinely used are intraepidermal, intradermal and patch testing (Demoly, Romano, & Bousquet, 2008).

Percutaneous or intraepidermal tests, also usually called skin prick test (SPT) or prick-puncture test, were initially described by Lewis and Grant in 1924, although Dr. Blackley in 1865 was the first to carry out a skin test on himself with pollen from *Lolium perenne*. In the 1970s, Pepys developed and standardized the performance of prick-puncture skin tests. The most common procedure is currently carried out by dropping a drop of the extract to be studied on the skin of the forearm and subsequently punctured with a sterile lancet with a tip length of 1.0 mm (Sanico, Bochner, & Saini, 2002). Some studies have also used the upper back as location for SPT (Bérot et al., 2020). An alternative variant also used is to first immerse the lancet in the well of a tray with the extract and then puncture the skin, applying the extract and prick in the same step. This variant has been extensively used in cases of food allergy studies, where the technique is also called prick-prick or prick by prick (Sanico et al., 2002).

Once the prick test is carried out, the skin is dried without rubbing, and then wait 15 min to see if there is reactivity in the puncture area. If there are specific IgE antibodies on the surface of cutaneous mast cells, a wheal and erythema occur at the puncture site since histamine release begins at 5 min and peaks at 30 min. SPT are safely used in the case of inhalants, foods, drugs, or insect stings.

Intracutaneous or Intradermal tests (*IDT*) are also used for the diagnosis of allergic diseases mediated by IgE or type I hypersensitivity. In these cases, their use is more restricted due to the higher percentage of false positives (Gorevic, 1997). The usual procedure employs insulin syringes loaded with the extract and, with the bevel of the needle facing upwards at an angle of 45°, an amount of between 0.01 and 0.05 mL is administered without inserting the entire needle (Ansotegui et al., 2020). The allergen concentration used in this method is usually 1/100 or 1/1000 of that used for SPT. The immediate reading of the tests for the study of type I hypersensitivity reactions is like that of the prick tests, around 15–20 min after the injection (Demoly et al., 2008). They are used in allergy studies in cases where prick tests are negative, mainly in studies with drugs, Hymenoptera venom and very occasionally with moulds.

In the case of drug allergy studies, skin tests with immediate reading to beta-lactamic antibiotics, and specifically, to penicillins, have an extraordinary performance (Romano et al., 2020), and are commonly used in allergy services (Castells, 2018). The use of skin tests with other antibiotics has a slightly lower performance but they are a fundamental pillar in diagnosing the diseases (Romano & Caubet, 2014). The intradermal tests also have major relevance to allergy from chemotherapeutic agents such as platins (Caiado et al., 2013).

When the patients have suffered non-immediate reactions and the suspect pathogenic mechanism is a T-cell mediated hypersensitivity (Lerch & Pichler, 2004; Rozieres, Vocanson, Saïd, Nosbaum, & Nicolas, 2009), skin IDT is also useful (Joshi & Khan, 2021), but more controversial (Phillips et al., 2019). In those cases, the late reading of IDT can be delayed from 12 h to several days later (Romano et al., 2004). In some diseases such as acute generalized exanthematous pustulosis (AGEP), Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN) (Iriki et al., 2014), the reading of the skin tests must be done at 24–48 h or even 1 week after IDT. In these disorders, the presence of effector-memory T cells and intraepidermal CD8(+) T cells with the local production of interferon gamma after the introduction of the triggering agent (Mizukawa et al., 2002) but also the presence of skin resident memory T cells have been shown (Tokura et al., 2021).

Patch tests are diagnostic tools mainly in contact dermatitis. But also, non-immediate drug reactions like those previously named AGEP, SJS and TEN, drug reactions with eosinophilia and systemic symptoms (DRESS) (Cabañas et al., 2020), single organ diseases and fixed drug exanthema (FDE) (Patel, John, Handler, & Schwartz, 2020), could also benefit from the use of a skin patch test for effective diagnosis (Copaescu, Gibson, Li, Trubiano, & Phillips, 2021). In all those conditions, a late reading of at least 48 h or more (Bhujoo et al., 2021) would give us a picture similar to that seen in contact dermatitis and would approximate a true etiological diagnosis (Belsito, 1989) with T cell involvement (Adam, Pichler, & Yerly, 2011).

4 *In vitro* methods to measure immune responses after SARS-Cov-2 infection

The human immune system makes antibodies and produces different lineages of T cells in response to SARS-CoV-2 infection. The early prediction of disease progression could be of help to assess the optimal treatment strategies, and an integrated knowledge of T-cell and antibody responses is urgently needed to find out biomarkers to monitor the COVID-19 disease. The development of reliable tests to detect those different arms of the immune response has been the objective of many research groups. In this review we aim to summarize the general aspects of the more relevant tests used in the clinical setting and to understand the advantages of the delayed type hypersensitivity (DTH) skin test, a simple and very informative method to measure immune T cell responses after SARS-CoV-2 infection and after vaccine administration.

4.1 SARS-CoV-2 protein description

The description of the relevant composition of the virus is important to understand the immunological targets that could be used to investigate the immune response generated after virus exposure and to study the immunogenicity of different vaccine approaches.

SARS-CoV-2 is a member of the family Coronaviridae and order Nidoviridiae. This family comprises two subfamilies, Coronavirinae and Torovirinae and

members of the subfamily Coronavirinae are subdivided into four genera: (a) Alphacoronavirus contains the human coronavirus (HCoV)-229E and HCoV-NL63; (b) Betacoronavirus includes HCoV-OC43, Severe Acute Respiratory Syndrome human coronavirus (SARS-HCoV), HCoV-HKU1, and Middle Eastern respiratory syndrome coronavirus (MERS-CoV); (c) Gamma coronavirus includes viruses of whales and birds and; (d) Delta coronavirus includes viruses isolated from pigs and birds (Burrell, Howard, & Murphy, 2016). SARS-CoV-2 belongs to Beta coronavirus together with two highly pathogenic viruses, SARS-CoV and MERS-CoV. SARS-CoV-2 is an enveloped and positive-sense single-stranded RNA (+ssRNA) virus (Kramer, Schwebke, & Kampf, 2006). The new coronavirus shares about 82% of its genome with SARS CoV-1 and both coronaviruses also share the same cellular receptor, which is the angiotensin-converting enzyme 2 (ACE2) (Gheblawi et al., 2020).

The proteins of SARS CoV consist of two large polyproteins: ORF1a and ORF1ab, four structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N), and eight accessory proteins: ORF3a, ORF3b (NP_828853.1, not present in SARS CoV-2), ORF6, ORF7a, ORF7b, ORF8a, ORF8b, and ORF9b (NP_828859.1, not present in SARS CoV-2) (Liu, Fung, Chong, Shukla, & Hilgenfeld, 2014). The non-structural proteins (nsps) are involved in virus processing and replication, while the structural proteins help in the assembly and release of new viral copies. The structural proteins produced are, *e.g.*, spike (S) protein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein. The M protein is the most abundant, while the E protein is the smallest in size among all the four structural proteins. More specifically, the M protein acts as a central organizer in assembling and shaping the viral envelope by interacting with other structural proteins. It binds with S and N proteins for the completion of new viral assemblies. The E protein is abundantly expressed in the replication cycle in the infected cells, although a small portion of it is incorporated into the viral envelope and mainly contributes to the viral assembly and budding. The N protein exhibits its functions by interaction with the positive RNA strand of the viral genome, thereby forming a helical ribonucleocapsid complex. It also interacts with other structural membrane proteins during the assembly of virions (Papageorgiou & Mohsin, 2020).

The envelope spike (S) protein receptor binding domain (RBD) of SARS-CoV-2 was shown to be structurally similar to that of SARS-CoV, despite amino acid variation at some key residues. In general, the spike protein of coronavirus is divided into the S1 and S2 domain, in which S1 is responsible for receptor binding and S2 domain is responsible for cell membrane fusion. The S1 domain of SARS-CoV and SARS-CoV-2 share around 50 conserved amino acids (Lu et al., 2020).

4.2 Understanding the adaptive immune response in covid19 patients

During the early stages of the pandemic, there was a clear focus on the development of methods to detect humoral immune responses to SARS-CoV-2. For most acute viral infections and following vaccination, seroconversion and the presence of

neutralising antibodies (Abs) is a clear functional correlate of immunity (Zinkernagel & Hengartner, 2006). However, the point-of-care (POC) serological tests to define SARS-CoV-2 exposure and presumably immunity that were introduced in the market at the beginning of the pandemic performed poorly (Baumgarth, Nikolich-Žugich, Lee, & Bhattacharya, 2020). These POC tests had poor specificity and sensitivity and therefore, many unreliable results confounded the true rate of positives after infection. Moreover, many of these POC tests intended to distinguish the time of infection, assuming that production of IgM would be equivalent to an ongoing infection and that an IgG positive test result would represent a much later stage of an active infection or a past infection and convalescence status.

Soon after the introduction of conventional ELISAs to measure different isotypes and neutralising Abs, it was clear that the performance data of these new generation tests to detect humoral immune responses were more accurate and their results better reflect the immune status of the investigated individuals. One critical point in the development of reliable methods was to identify the immunodominant antigen that drives the humoral immune responses in SARS-CoV-2 infected individuals (Piccoli et al., 2020). Although there are several candidates, the more obvious were the spike and nucleocapsid proteins. Nucleocapsid and spike IgG titres are highly correlated (Piccoli et al., 2020). The spike protein is the target of SARS-CoV-2 neutralising antibodies and RBD of the spike protein is the target of >90% of neutralising antibodies in COVID-19 (Premkumar et al., 2020). Serological studies have found that, as expected, high antigen viral load results in higher antibody titres. Neutralising and total anti-spike antibodies correlate with severe disease (Premkumar et al., 2020).

It was evident that, after optimization of the conventional ELISAs, there was an urgent need for the study of the cellular immune responses in SARS-CoV-2 infection. Antibodies can be a useful surrogate marker of CD4+ T cell responses in many infections and that is one of the reasons why antibody assays, which are more practical to perform in large cohorts of patients and easier to handle, are the first investigated part of the immune responses. However, there are some reasons to believe that in SARS-CoV-2 infection these antibody titres are poor predictors on the development of specific anti-SARS-CoV-2 CD4+ T cells and to some extent this could be explained because of immunological differences in the pattern of antigen immunodominance in T cell responses (M, spike, and N proteins co-dominant) (Grifoni et al., 2020). One special situation is the asymptomatic cases of SARS-CoV-2 individuals where there is a tendency to have low or undetectable antibodies but high numbers of circulating T cells showing that early T cell responses could result in absence of clinical disease symptoms (Sekine et al., 2020). This also could be explained by the fact that among the best-known risk factors for severe disease is the age of the infected person. Older individuals are less likely to make a coordinated adaptive immune response to SAR-CoV-2 (Grifoni et al., 2020).

4.3 *In vitro* methods to measure SARS-CoV-2 cellular immune responses

In general terms, the evaluation of population immunity is based on antibody detection studies. However, in the context of evidence for cellular responses in seronegative exposed individuals (Gallais et al., 2021) and the potential waning of antibody responses over time (Ojeda et al., 2021; Shrotri et al., 2021), current surveillance methods are likely to be underestimating both exposure and immunity. Moreover, CD4+ and CD8+ T cells targeting structural viral proteins appear to confer broad and long-lasting protection against SARS-CoV (15) (Liu et al., 2017). Thus, a better understanding of the role of T cells in the long-term protection from COVID-19 is crucial in estimating population-level immunity, vaccine development, and long-term surveillance of vaccine efficacy (Dan et al., 2021).

Extensive research has been conducted to identify epitopes involved in T-cell responses, including a large study of overlapping peptides spanning the entire SARS-CoV-2 proteome (Grifoni et al., 2020), showing that spike protein epitope pools are the most immunogenic stimuli (Aiello et al., 2021; Murugesan et al., 2020).

Different assays have been used to measure SARS-CoV-2- specific T-cell responses, including intracellular cytokine staining, activation-induced markers and interferon gamma release assays (IGRAs). Although an extensive review of detailed protocols is out of the scope of this review, we will review the basic principle of the IGRA, which is the more common method employed in studies *in vitro*, in order to better understand the advantages and disadvantages of this assay compared to the DTH *in vivo* method.

Interferon gamma release assays (*IGRAs*). Interferon gamma is a Th1-type cytokine, induced upon stimulation of T cells by a specific-antigen, T-cell mitogens and some pharmacologic stimuli. IFN-gamma is synthesized by a CD4+Th1 helper subset, some CD8+ T-cell subpopulations, NK cells and activated macrophages. IFN-gamma release assays after T cell stimulation have been explored in several infectious diseases and cytokine release-based tests in whole blood are routinely or experimentally used for cytomegalovirus (CMV) infection monitoring (Kim, 2020), and have been explored for hepatitis B virus (Dammermann et al., 2015), toxoplasmosis (Mahmoudi, Mamishi, Suo, & Keshavarz, 2017) and cystic echinococcosis (Petrone et al., 2017, 2020) diagnosis. This approach has also been used to investigate SARS-CoV-2 T cell responses (Petrone et al., 2021). At this moment there are several commercially available options to investigate interferon gamma release methods in the COVID clinical setting. Whole IGRA blood assays are very convenient in terms of using them in a clinical laboratory outside of the more dedicated research facilities (Martínez-Gallo et al., 2021). One important aspect is to determine the specific response to peptides corresponding to proteins encoded by different viral genomic regions (spike, membrane, nuclear proteins or others), as well as the determination of optimal concentrations and read-out that have been employed in the

different available methods, because differences in the published results could correspond to methodological details. In conclusion, IGRAs are an important diagnostic tool in COVID infected and vaccinated individuals but, in order to expand its use, some uncertainties about the cut-off values and the real-life correspondence with protection from infection needs to be further defined to augment the prognostic value of a positive IGRA result.

As explained before, the Mantoux tuberculin skin test (TST) was developed early in the 20th century, and although with some drawbacks, it is still present in the actual medical practice (Richeldi, 2009). During the last few decades it has been used in parallel with the IGRA tests, producing an enormous amount of data permitting the comparison of both methods (Hass & Belknap, 2019). All these data have been used to improve the diagnosis of latent tuberculosis infection, where the performance of IGRA tests have been demonstrated to be superior in terms of discriminating infection in vaccinated individuals (Lewinsohn et al., 2017). This has been achieved using IGRA tests with peptides not present in the vaccine formulation that are not possible to distinguish in the TST test.

However, the analysis of the situation in SARS-CoV-2 infection is completely different. In these COVID patients the focus of the results should be the sensitivity of the test, finding as many individuals as possible that either by natural exposure or after vaccination could have developed an adaptive T cell response. In that scenario an *in vivo* presentation of the antigen to the T-cell population could be superior if these two methods are compared (see Table 2 to understand differences exhibited between IGRA and TST).

Table 2 Comparison of DTH skin test and interferon-gamma release assays.

	DTH	**IGRA**
Advantages	– Low cost – Easy to perform – Ability to test a large number of individuals in a short-period of time – Could be single visit if results are sent by phone – Electronic results report	– Single visit – Positive and negative controls into the test – Objective results – Electronic laboratory report – Use of selected peptides
Disadvantages	– More subjective-reader variability – Influence of boost phenomenon	– Higher cost for reagents – Blood extraction facilities – Handle of sample – More difficult technical laboratory phase
Common	– Affected by immunocompetence of individuals	– Affected by immunocompetence of individuals

5 A novel application of a DTH method to measure immune responses after SARS-CoV-2 infection

During the critical months of the COVID pandemic situation, it was necessary to think in some practical approach to diagnose SARS-CoV-2 exposure in immunocompromised individuals that were not capable of producing antibodies due to their subjacent immunological defect. As humoral immunodeficient patients are the most frequent presentations of primary and secondary immunodeficiency situations, there was a lack of specific tests to analyse this subgroup of COVID -infected individuals using the conventional ELISAs developed at that time. To overcome this, our PID clinical group (Barrios et al., 2020) developed a COVID-specific skin test based on RBD of the spike protein of SARS-CoV-2 (CoviDCELL®). All the research was conducted after the protocol was approved by the ethical committee of the Hospital (CHUC_2020_92) and in accordance with the requirements expressed in Law 737/2015 about biomedical research and the Declaration of Helsinki (revised Brasil, October 2013).

Briefly, 25 μL (0.1 mg/mL final concentration) of a RBD commercially available protein (Vitro, Spain) was injected intradermally *via* a 25-gauge needle in the ventral part of the arm. The final concentration used was similar to the concentration used in the tuberculin test (Badaro et al., 2020). A *Candida albicans* extract was used as a control reaction to assess cellular immune competence of the participants. All antigen dilutions were made under sterile conditions. Patients were instructed to send a photo with the skin test reaction after 15 min and 6, 12, 24 and 48 h after injection. The protocol was performed according to usual clinical practice and following the Allergology Procedures Manual and the Safety and Quality Recommendations in Allergology (RESCAL-2018) of the *Spanish Society of Clinical Allergology* (SEAIC) to carry out allergology procedures (E2). According to the manual, intra-epidermal and intradermal skin tests are at Level A defined as the "set of tests that meet the following criteria of low complexity, short duration (the patient must remain under observation for less than 2 h) and, finally, low risk of reaction." The tests were carried out in the area of diagnostic techniques of the Allergy Service according to the usual clinical practice. Intradermal tests were not performed in patients with a history of grade II or higher anaphylaxis. Erythema and indurations were registered. A skin test (spike and candida-DTH) was considered positive if the area of erythema was greater than 10 mm at least after 6 h. The first group of results were obtained from CoviDCell tests performed in healthy individuals (not exposed to SARS-CoV-2 virus) or in immunocompetent-infected individuals (Barrios et al., 2021). In these immunocompetent individuals, testing for IgG-specific anti-RBD antibodies (*Euroimmun*, Lübeck, Germany) was also performed and the results correlated with the skin test outcomes.

Shown in Fig. 2 is a representative set of skin reactions obtained in this group of exposed immunocompetent individuals. As described with the tuberculin DTH test there is some variability in the biologic response according to the stratification of the

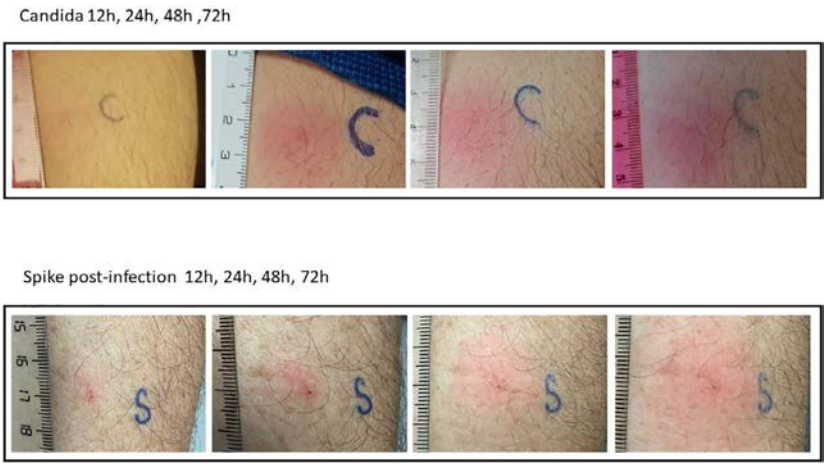

FIG. 2

Upper: example of response to Candida (C) antigen after intradermal test in a subject at 12, 24, 48 and 72 h. Below: example of response to Spike (S) antigen after intradermal test in a subject with a fully recovered SARS-CoV-2 disease at 12, 24, 48 and 72 h.

patients into different groups depending on their clinical presentation. From this set of tests, it was evident that DTH using RBD derived S protein from SARS-CoV-2 is a simple method to investigate immunity after virus exposure. The DTH test that was performed in a group of non-exposed individuals was negative in all of the cases, showing a high specificity of the test. Moreover, comparisons between specific anti-RBD IgG and spike-DTH cutaneous test results, to identify the exposed individuals, showed a concordance number of 84.3%. In this set of exposed individuals, CoviDCELL® showed a superior capacity to identify exposed individuals.

Although until this manuscript has been written, there were no more scientific public data regarding the use of DTH to assess immune exposure to SARS-CoV-2, several biotech companies have announced the use of peptide-base or protein-derived moieties from different parts of the virus with the intended use of developing commercial delayed type hypersensitivity tests to assess T-cell immunity after infection or to measure immunogenicity elicited by the vaccines. One of the concerns regarding the anti-COVID-19 DTH reaction is the performance of the test with the new variants produced by the virus. In this regard, ELISAs can be more susceptible to changes in the conformational epitopes recognized by the patient's antibodies. But T-cells recognize many linear epitopes, and a substantial number of changes in the potential epitopes between the variants would be required to affect the T-cell recognition. These facts suggest that the skin test will be relatively unaffected by the virus variants.

6 DTH to measure immunogenicity elicited by covid vaccines

The same protocol of CoviDCELL was implemented to investigate immunogenicity produced after vaccination (Barrios et al., 2021). For this study, prioritized health care workers group vaccinated at the Hospital Universitario de Canarias and vaccinated with the BNT162b2 mRNA Pfizer vaccine were offered to participate in the study approved by the ethical committee of the Hospital (CHUC_2021_04).

The results showed that the CoviDCELL test was positive in 73% of the individuals after a single dose of vaccine. All participants developed a positive DTH test after two doses of the vaccine, showing that both vaccine doses are needed for the detection of an *in vivo* T cell immune reaction. The kinetics of the DTH skin reaction was more rapid (in 12 h) and wanes faster (in 48 h) in vaccinated individuals compared with naturally immunized patients due to a prior infection. Fig. 3 shows a representative set of skin reactions obtained in this group of vaccinated immunocompetent individuals.

These results demonstrate that the DTH test is an affordable and simple method that could help in the future to answer basic immunogenicity questions on large-scale population vaccine studies.

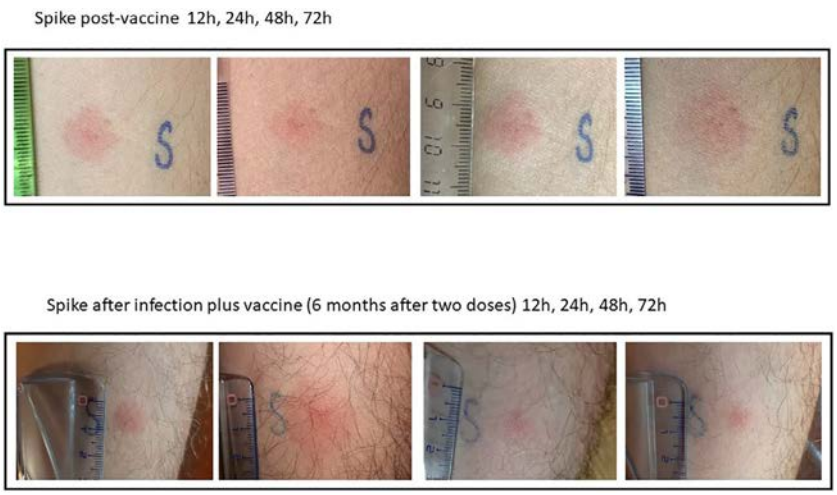

FIG. 3

Upper: example of response to Spike (S) antigen after intradermal test at 12, 24, 48 and 72 h in a fully vaccinated subject with a mRNA vaccine. Below: example of response to Spike (S) antigen after intradermal test at 12, 24, 48 and 72 h in a subject with a fully recovered SARS-CoV-2 disease and two doses of a mRNA vaccine.

7 Future prospects of DTH to study SARS-CoV-2 immunogenicity

At the moment of writing this review, our clinical group have submitted results obtained in vaccinated immunodeficient patients and vaccinated kidney-transplant patients for publication. Also, we have conducted a 6-month follow-up DTH study of immunocompetent health care workers to address the question of "For how long will the immunogenicity provided by the vaccines last?".

Many biological aspects of the test need to be further investigated. For example, the influence of the diameter and kinetics of the reaction in determining the distinction between natural *vs* vaccinated immune responses, the correspondence with the IGRAs available to improve the cut-off definition of positive tests and also the interesting possibility of a boost influence of the intradermal injection in the sequential analysis of the same individuals (an initial negative DTH test primes the immune system so that a subsequent test becomes positive). There are also many technological details that could be addressed in the next several months like the digital treatment of the images but also to work on automatic algorithms that allow the processing of large amounts of data that could be necessary to handle in immunogenicity studies conducted in large populations. Properly designed and powered longitudinal studies will provide insights in these areas.

It is evident that an old test could still compete in the forefront repertoire of methods that will allow us to know better this new challenge of the SARS-CoV-2 pandemic. The relevance of this work is the revision of methods more imaginative and simpler, to afford the tremendous challenge of this pandemic in the coming years.

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Victor Matheu and Yvelise Barrios have registered (50%) CoviDCELL® 202199800 402351.

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