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(54) Title: METHODS AND SYSTEMS FOR PREDICTING ALLERGIC RESPONSE

(57) Abstract: The present invention provides systems and methods for predicting an allergic response in a subject by measuring the amounts of RNA species from B cells that encode at least a part of the Immunoglobulin E (IgE) constant region (Ce), such as nonproductive epsilon germline transcripts (eGLTs).

METHODS AND SYSTEMS FOR PREDICTING ALLERGIC RESPONSE

Technical Field

The invention generally relates to the fields of medicine, allergies, and immunology, and, more particularly, to measurement, monitoring, and prediction of allergy.

Background

Allergy is estimated to affect 30% of the global population. The prevalence of diagnosed allergies is continually increasing due to numerous factors, but in part by the recognition of new allergens and allergic responses as well as the increased availability of allergy tests.

Allergies are characterized by a number of conditions caused by hypersensitivity of the immune system to otherwise harmless substances in the environment. In general, an allergic reaction occurs when aspects of the immune system overreact to the presence of a substance (an allergen) that, absent the allergy, would not cause a reaction. Allergies have a negative impact on individuals' quality of life and can lead to societal and personal economic burdens. As mere exposure to certain allergens can have life-threatening consequences, people suffering from allergies are often required to be hypervigilant and forced to alter their behavior to avoid allergens.

As generally understood, an allergen is a type of antigen that produces an abnormally vigorous immune response in which the immune system combats a perceived threat that would otherwise be harmless. In technical terms, an allergen is an antigen that is capable of stimulating a type I hypersensitivity reaction mediated by Immunoglobulin E (IgE) in atopic individuals. Most humans mount significant IgE responses only as a defense against parasitic infections. However, some individuals may respond to common environmental antigens. This hereditary predisposition is called atopy. In atopic individuals, non-parasitic antigens stimulate inappropriate IgE production, leading to type I hypersensitivity.

Emerging research has shown that allergies are not homogenous conditions. Allergy research has shown that many common allergies are more complex than previously thought. For example, patients with peanut allergies may be allergic to one or more proteins found in peanuts

as well as one or more epitopes of said proteins. Further, people with a particular allergy can produce different IgE antibodies in response to the same antigen.

There are several methods for testing current allergic status in human patients. However, existing tests lack the ability to predict future allergic status.

For example, ELISA-based serum IgE testing is among the most common forms of allergy test. However, such tests are low-throughput and suffer additional drawbacks. Tests using this antibody-based method can require unique capture antibodies for every allergen tested. For example, different capture antibodies may be required to capture the different proteins and/or identify protein epitopes associated with a particular food allergy. Moreover, certain allergens can cross-react with different capture antibodies on an ELISA panel, which obfuscates the results. Thus, the breadth of antigens tested by any single panel is limited.

Another common form of allergy testing includes directly contacting a patient with an allergen, e.g., using skin prick tests, patch tests, intradermal tests, or oral food challenges. These tests pose the clear problem of directly contacting individuals with an allergen, which can lead to discomfort or more severe allergic responses. The risk of severe reactions necessarily means that tests for newly-discovered allergens undergo rigorous safety testing, which means allergy testing can lag allergen discovery. Further, extracting allergens for use in this manner of testing requires stringent and expensive manufacturing protocols to ensure high purity and the absence of contamination. Additionally, although contact testing has a high sensitivity, its specificity can be low.

Summary

The present invention provides systems and methods for predicting an allergic response in a subject by measuring RNA from B cells that encode at least a part of the Immunoglobulin E (IgE) constant region (Cε), including nonproductive epsilon germline transcripts (εGLTs).

The present Inventors have determined that germline transcripts from antibody heavy chain constant region loci within B cells are predictive of future antibody class production. Those transcripts can be identified and measured to determine the propensity of B cells to undergo antibody class switch recombination to produce, for example, IgE antibodies. Thus, germline transcription is useful for predicting future allergic responses, including predisposition to allergy, and therefore leads to potential intervention.

Notably, the present invention allows accurate prediction of allergy predisposition for myriad potential allergens. Further, the presently disclosed methods and systems are useful for retrospective study of allergy development and the longitudinal dynamics of allergic status. Methods and systems of the invention are useful to predict which specific antigens from a source containing a plurality of antigens are most likely to cause an allergic response. Further, the systems and methods described herein allow accurate prediction of an intervention's therapeutic efficacy.

Thus, the present invention includes methods for predicting an allergic response. In certain methods, a body fluid or tissue sample including one or more RNA species from B cells encoding all or a fragment of an Immunoglobulin is used to predict an immunity profile for the individual from whom the sample was obtained. In a preferred embodiment, the immunoglobulin is an IgE or IgG immunoglobulin. In a highly-preferred embodiment, the fragment is an IgE or IgG heavy chain constant region. The identity of the RNA species is then determined. The types of RNA obtained and the amount thereof are used to predict an allergic response in the subject, for example, when the measured amount exceeds a predetermined threshold.

In certain methods, the threshold is determined by measuring the amounts of one or more RNA species in patients with a known allergy. The amounts measured in the patients may be taken at any time, including after the patients experience an allergic response.

Predicting an allergic response can include predicting the likelihood of developing an allergy without determining the allergen causing the predicted response. Predicting an allergic response can also include identifying at least one allergen that causes the predicted allergic response in the subject. Identified allergens can include, for example, at least one of a food allergen, a plant allergen, a fungal allergen, an animal allergen, a drug allergen, a cosmetic allergen, and a latex allergen.

The RNA species can include at least part of a nonproductive germline transcript (GLT). Thus, in certain methods, the steps of identifying and/or measuring includes assessing GLT expression in the sample. The GLT can contain one or more IgE constant region exons and thus, as an epsilon germline transcript (εGLT), include a nucleic acid sequence spliced to the 5' IgE constant region exon. The nucleic acid sequence spliced to the 5' IgE constant region exon can be from the Iε-exon or the switch region (Sε). In certain methods, identifying the one or more

RNA species includes determining the presence of one or more splice junctions spanning the 5' IgE constant region exon and the Iε-exon or the switch region (Sε) in one or more εGLTs.

In certain aspects, the invention further comprises sequencing the one or more RNA species. The sequencing may include single-cell RNA-sequencing. In certain methods, identifying and/or measuring includes reverse transcription-quantitative PCR.

Brief Description of the Drawing

FIG. 1 shows the production of nonproductive epsilon germline transcripts (εGLTs).

Detailed Description

The present invention provides systems and methods for predicting an allergic response by measuring the expression of one or more RNA species from B cells. In preferred embodiments, the RNA encodes at least a part of the Immunoglobulin and preferably an immunoglobulin E (IgE) constant region (Cε), such as nonproductive epsilon germline transcripts (εGLTs).

IgE antibodies mediate the allergic response by binding to specific receptors on inflammatory immune cells, such as mast cells in mucosal tissues lining body surfaces and cavities, as well as basophils in the circulation. Those cells mediate allergic responses triggered by specific antigens (allergens) that are recognized by IgE through the release of inflammatory molecules, such as histamine. The inflammatory response leads to symptoms, such as sneezing, runny or stuffed nose, itchy eyes, breathing difficulties, and, in extreme cases, anaphylactic shock and even death.

Class switch recombination (CSR) is the DNA recombination process that enables switching of antibody class, and thus effector function, while maintaining the specificity of the antibody as determined by its variable region. Class switch recombination to IgE involves the replacement of an antibody heavy chain constant region gene segment with an IgE constant region (Cε) gene segment. Prior to CSR to IgE, a B cell will express a nonproductive, epsilon germline transcript (εGLT) which is the product of transcription beginning upstream of the Iε-

exon and proceeding through the switch region (Sε) and exons encoding the Cε. Splicing of the GLT produces an intronic lariat as well as a mature polyadenylated transcript that canonically features the Iε-exon spliced to the 5' Cε exon.

FIG. 1 diagrams the organization of the immunoglobulin heavy chain locus of a B cell expressing an IgM antibody prior to CSR to IgE. For clarity, only regions pertaining to IgE CSR are shown. A primary epsilon GLT is produced by transcription, which begins at a promoter element (arrow in FIG. 1) upstream of the Iε-exon and proceeding through the Cε exons. Splicing of this product yields one or more mature εGLTs that consist of the 5' Cε exon spliced to a unique upstream splice donor, such as the canonical Iε-exon (εGLT 1). A primer pair spanning such a splice junction can specifically amplify an εGLT.

The inventors have discovered that an assessment εGLT expression can be used to predict an allergy response and/or allergy status in an individual.

Any body fluid or tissue sample is appropriate for use in the invention. Preferably, a sample is obtained that is a source of B cells. A sample including B cells may be, for example, blood, saliva, sputum, urine, semen, transvaginal fluid, cerebrospinal fluid, sweat, stool, a cell or a tissue. Preferably, the sample is peripheral blood. Methods of the invention may further include enriching or isolating B cells from the sample. Methods of isolating or enriching B cells are known and provided, for example, in Croote et al., "Human IgE producing B cells have a unique transcriptional program and generate high affinity, allergen-specific antibodies", bioRxiv 327866; doi: https://doi.org/10.1101/327866 (2018) and Croote et al., "High-affinity allergen-specific human antibodies cloned from single IgE B cell transcriptomes", *Science*, 362, 1306-1309 (2018), each of which is incorporated herein by reference. In certain methods and systems, B cells are isolated or enriched using cell sorting flow cytometry, such as through fluorescence-activated cell sorting methods. In certain aspects, the B cells are enriched for certain B cell isotypes or certain B cell subsets.

In certain aspects, the B cells may be lysed to release the RNA. After release, the RNA may be isolated, for example, with an RNA isolation kit, such as the RNA isolation kit sold under the trade name RNeasy by Qiagen (Valencia, CA). The isolated RNA may be used to generate cDNA for analysis. Alternatively, the RNA may be used directly in the generation of cDNA without a dedicated isolation step. The generation of cDNA can be done using a variety of methods known in the art. The cDNA can be used to create a cDNA library for further analysis.

Methods of the invention further include identifying the RNA species and measuring the amounts of RNA in the sample. Such methods are known in the art and may include one or more methods of quantifying expression levels via single-cell or bulk RNA-Seq, RT-qPCR, direct RNA sequencing, or in-situ hybridization. Other techniques for identifying RNA include immune cells staining, immune fluorescence, flow cytometry, and sequencing. Single cell RNA detection methods (e.g., RNASeq) are useful in the context of the invention but not necessary, as the invention has the advantage of lower cost, lower complexity, and higher throughput in bulk cell measurements.

Certain methods comprise generation of cDNA. The generation of cDNA can be done using a variety of methods. For example, the cDNA can be generated using a reverse transcriptase, which has the ability to use the information in a molecule of RNA to generate a molecule of cDNA. A reverse transcriptase is an RNA-dependent DNA polymerase. Like all DNA polymerases it cannot initiate synthesis *de novo* but depends on the presence of a primer. Since many RNAs have a poly-A tail at the 3' end, oligo-dT is frequently used to prime DNA synthesis. It is also possible to generate cDNAs by using either random primers or primers designed to amplify a specific RNA. Once a first strand of cDNA has been created, it is generally necessary to produce a second strand of DNA. A person of skill in the art will recognize that there are many methods for producing the second strand. One mechanism involves exposure of the DNA/RNA hybrid to a combination of RNAase-H and DNA polymerase. RNAase-H has the ability to cause single-stranded nicks in the RNA, and DNA polymerase can then use these single-stranded nicks to initiate "second strand" DNA synthesis. This two-step procedure has been optimized to maximize fidelity and length of cDNAs. Following second strand synthesis, the cDNA may be amplified by PCR.

Biotinylated capture baits or probes can be used for the targeted enrichment of specific cDNA molecules of interest. The biotinylated capture probes may comprise RNA, DNA, or a hybrid of RNA and DNA nucleotides. Biotinylated RNA capture probes may be added to the cDNA library and incubated for a period of time and at a temperature sufficient for the biotinylated RNA capture probes to hybridize to their target molecules of cDNA based on Watson-Crick base pairing. For example, the mixture containing cDNA and probes may be incubated at 65 degrees Celsius for 24 hours. After hybridization, the biotinylated RNA capture

probes that are hybridized with the target cDNA molecules may be captured and segregated using streptavidin or an antibody. The target cDNA molecules can then be amplified by PCR.

Library preparation is used to modify cDNA so it is compatible with the requirements of the sequencing instrument to be used. During ligation or other reactions, such as tagmentation, full or partial adapters may be incorporated into the cDNA. Preferably, the adapters may comprise sequences recognized by a sequencing platform, such as the Illumina P5/P7 (flow cell binding sequences). The adapters may also comprise PCR primer biding sites for amplifying the cDNA library. In some embodiments, the adapters may further include barcode sequences. The barcode sequences may be used to give each library in a pooled sequencing run a unique tag or identifier.

Sequencing is used to determine the nucleic acid sequence of molecules within a sequencing library. An example of a sequencing technology that can be used is Illumina sequencing. Illumina sequencing is based on sequencing by synthesis. DNA on a solid surface is amplified using fold-back PCR and anchored primers. Four fluorophore-labeled, reversibly terminating nucleotides are then used to perform sequential sequencing. After nucleotide incorporation, a laser is used to excite the fluorophores, an image is captured, and the identity of the base is recorded. Sequencing according to this technology is described in U.S. Pub. 2011/0009278, U.S. Pub. 2007/0114362, U.S. Pub. 2006/0024681, U.S. Pub. 2006/0292611, U.S. Pat. 7,960,120, U.S. Pat. 7,835,871, U.S. Pat. 7,232,656, U.S. Pat. 7,598,035, U.S. Pat. 6,306,597, U.S. Pat. 6,210,891, U.S. Pat. 6,828,100, U.S. Pat. 6,833,246, and U.S. Pat. 6,911,345, each incorporated by reference. Sequencing may include single-cell sequencing methods such as single cell RNA sequencing (scRNA-Seq) methods, whereby cells may be isolated in partitions prior to lysis and cDNA synthesis.

After sequencing, the RNA species in the sample can be identified, for example, by aligning the resulting sequence reads to a reference. When the RNA species include εGLTs, alignment may be to the GRCh38 human genome, preferably using splice-aware alignment software, such as STAR (A Dobin et al., "STAR: ultrafast universal RNA-seq aligner", *Bioinformatics*, 29, 15-21 (2013)), which is incorporated herein by reference. The Inventors have discovered that identifying one or more unique εGLTs in a sample is accomplished by identifying the presence of splice junctions spanning the 5' IgE constant region exon and either the Iε-exon or a location within the Sε.

In certain aspects, the amounts of particular RNA species in a sample are measured using sequencing. For example, relative expression may be calculated based on normalized gene counts or sequencing reads mapping to splice junctions. Additionally, cDNA from the RNA species can be labelled with barcodes and/or unique molecule identifiers to ascertain their relative expression levels.

In certain aspects, the RNA species, such as εGLTs, are identified and/or measured by RT-qPCR. In certain aspects, the RT-qPCR is one- or two-step RT-qPCR.

Methods of the invention further include predicting an allergic response. For example, methods may include predicting a subject's predisposition to experiencing an allergic response, predicting a subject's predisposition to developing an allergy, predicting a subject's allergic status, predicting a subject's allergic reaction severity, and/or predicting the likelihood that a subject experiences alleviation or elimination of an allergic response by a particular therapeutic intervention.

Methods may include determining whether expression of one or more RNA species in the sample exceeds or falls below a particular threshold. The threshold may be based on RNA expression analyses of patients with known allergies. Individuals with a known allergy may have RNA species comprising a nucleic acid sequence encoding at least a part of the Immunoglobulin E (IgE) constant region (Cε) from their B cells identified and measured. The expression levels of the RNA species may be compared to individuals without the known allergic condition and/or compared from the same patient before and after exposure to an antigen. Based on the differences in these comparisons, expression patterns of one or more unique RNA species, such as εGLTs, can be associated with allergic status or a particular allergic condition. Using these expression patterns, thresholds can be set and used to predict allergic reactions in subjects to be tested.

In certain aspects, multiple thresholds can be used. For example, thresholds among different RNA species or within a single RNA species. The thresholds can be used, for example, to determine not only that a patient is or is not likely to experience an allergic reaction, but also with a particular confidence value.

Methods of the invention may also include determining the severity of a potential allergic reaction in a subject. In certain aspects, thresholds can be set to ascertain the potential severity and type of allergic response in a subject. Similarly, methods of invention can also be used to

determine the likelihood that a particular therapeutic intervention ameliorates or eliminates a particular allergic condition.

Since the invention does not require the use of antibody-based hybridization techniques, it can provide granular results. Not only can the propensity to develop an allergy be predicted, but in certain aspects, the specific allergen (e.g., protein or protein epitope) likely to cause an allergic condition can also be predicted. In certain aspects, RNA species containing an antibody variable region found in a sample are compared to an antibody database of variable regions with known specificities to predict reactivity to a particular allergen or epitope. As the present invention uses genetic information, it is not affected by the uncertainties endemic to hybridization assays using antibodies.

Similarly, since the presently disclosed systems and methods do not require the use of allergen extracts or antibodies, they can be used to concurrently test for a broad and diverse array of predicted antigen reactivities. For example, the systems and methods of the disclosure can predict an allergic reaction caused by specific allergens that may include, but are not limited to, a food allergen, a plant allergen, a fungal allergen, an animal allergen, a dust mite allergen, a drug allergen, a cosmetic allergen, or a latex allergen.

Some foods such as peanuts (a legume), nuts, seafood and shellfish are the cause of serious allergies in many people. Officially, the United States Food and Drug Administration recognizes eight foods as being common for allergic reactions in a large segment of the sensitive population. These include peanuts, tree nuts, eggs, milk, shellfish, fish, wheat and their derivatives, and soy and their derivatives, as well as sulfites (chemical-based, often found in flavors and colors in foods). Accordingly, the presently disclosed systems and methods can be used to predict an allergic response in a subject caused by a particular food or allergen found in a food.

An allergic reaction can be caused by any form of direct contact with the allergen—consuming food or drink one is sensitive to (ingestion), breathing in pollen, perfume or pet dander (inhalation), or brushing a body part against an allergy-causing plant (direct contact). The presently disclosed systems and methods can be used to predict an allergic response in a subject due to ingestion, inhalation, and/or contact with an allergen or source of allergens. Thus, the presently disclosed invention provides an obvious improvement over traditional contact-based

allergy test methods, which carry the risk of reactions and may not be available for all allergens to which a subject is exposed by ingestion or inhalation.

In certain embodiments, methods and systems of the disclosure may include comparing the expression analysis results of the subject to a database of expression profiles and patterns from individuals with known allergies. The database can be continually updated. In certain aspects, a subject's RNA species expression data can be reanalyzed as the database is updated. Thus, new allergic response predictions can be made without requiring the subject to provide an additional sample. Similarly, additional information regarding patients in the database, such as clinical outcomes, medical history, genetic/epigenetic information, comorbidities, responses to treatment, and the like can be compared with a subject receiving a test to provide a more wholistic prediction that can be used to inform treatment decisions.

Incorporation by Reference

References and citations to other documents, such as patents, patent applications, patent publications, journals, books, papers, web contents, have been made throughout this disclosure. All such documents are hereby incorporated herein by reference in their entirety for all purposes.

Equivalents

Various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including references to the scientific and patent literature cited herein. The subject matter herein contains important information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof.

What is claimed is:

1. A method for predicting an allergic response in a subject, the method comprising:

obtaining a sample from a subject comprising one or more RNA species that comprise a sequence encoding at least a part of the Immunoglobulin E (IgE) constant region (Cε);

identifying the RNA species in the sample;

measuring an amount of the identified RNA species in the sample; and

predicting an allergic response in the subject based on the amount of measured RNA species relative to a certain threshold.

2. The method of claim 1, wherein the predicted allergic response is a predicted predisposition to developing an allergy.

3. The method of claim 1, wherein the predicted allergic response is a prediction of allergic reaction severity.

4. The method of claim 1, further comprising the step of predicting alleviation or elimination an existing allergic response.

5. The method of claim 4, further comprising the step of determining an appropriate therapeutic intervention.

6. The method of claim 1, wherein the threshold is determined by measuring the amounts of the identified RNA species in patients with a known allergic condition.

7. The method of claim 1, wherein the predicting step comprises identifying at least one allergen that causes the predicted allergic response.

8. The method of claim 7, wherein the allergen comprises at least one of a food allergen, a plant allergen, a fungal allergen, an animal allergen, a drug allergen, a cosmetic allergen, and a latex allergen.

9. The method of claim 1, wherein the one or more RNA species is produced by germline transcription.

10. The method of claim 9, wherein the one or more RNA species comprises at least part of a nonproductive epsilon germline transcript (εGLT).

11. The method of claim 10, wherein the identifying and/or measuring step comprises assessing expression of one or more εGLTs in the sample.

12. The method of claim 10, wherein the εGLT comprises a nucleic acid sequence spliced to a 5' IgE constant region exon.

13. The method of claim 12, wherein the nucleic acid sequence spliced to the 5' IgE constant region exon is from a Iε-exon or a switch region (Sε).

14. The method of claim 13, wherein identifying the one or more RNA species comprises determining the presence in the εGLT of one or more splice junctions spanning the 5' IgE constant region exon and the Iε-exon or the switch region (Sε).

15. The method of claim 1, wherein the identifying and/or measuring step comprises sequencing the one or more RNA species.

16. The method of claim 1, wherein the identifying and/or measuring step comprises reverse transcription-quantitative PCR.

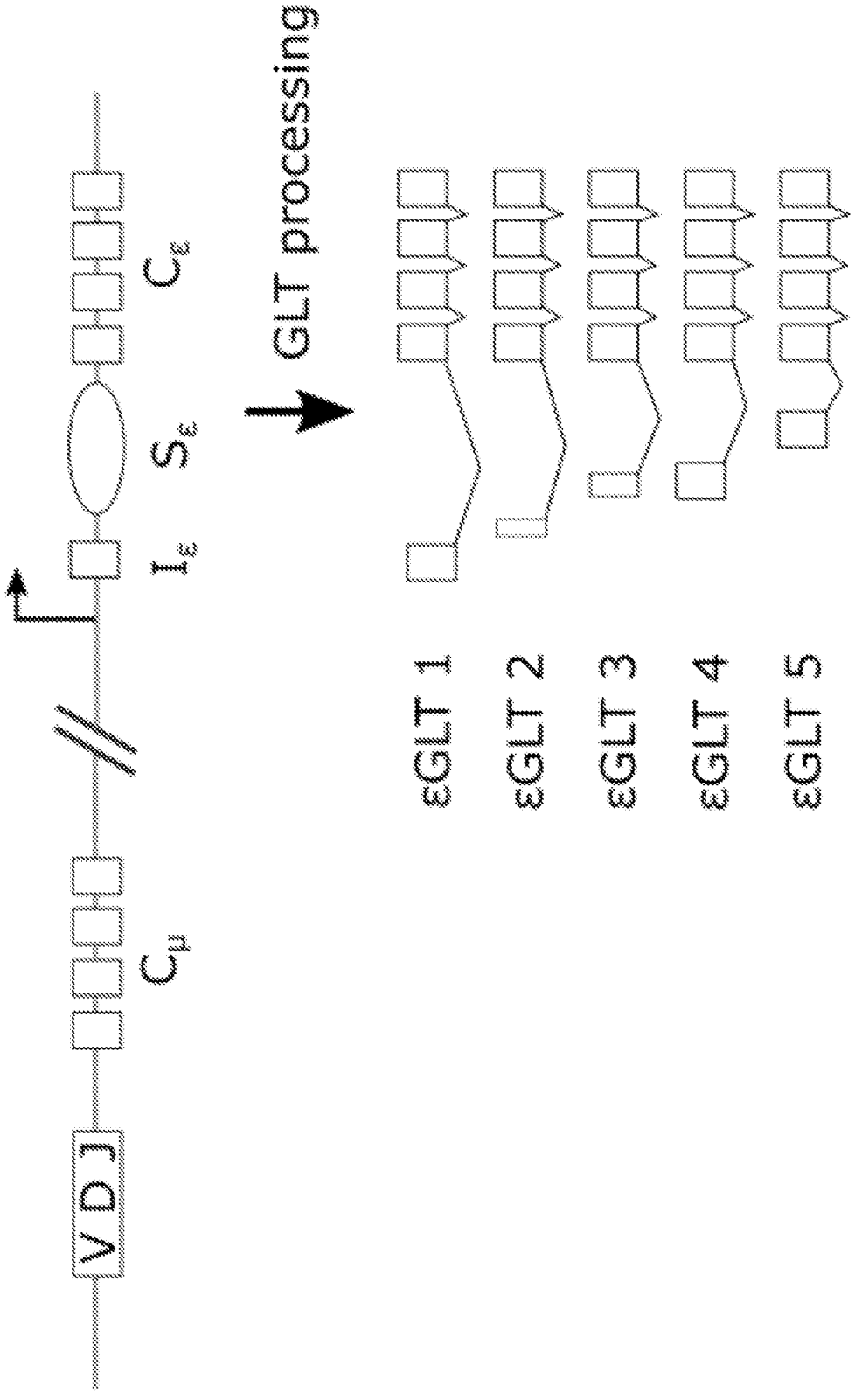
VDJ
C_μ
I_ε
S_ε
C_ε
GLT processing
εGLT 1
εGLT 2
εGLT 3
εGLT 4
εGLT 5

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 22/19880

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12Q 1/68; G01N 33/53; G01N 33/50 (2022.01)

CPC - G01N 33/5047; G01N 33/30; G01N 33/5047; C12Q 2600/112; G01N 2800/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	TAKHAR et al. Allergen Drives Class Switching to IgE in the Nasal Mucosa in Allergic Rhinitis. The Journal of Immunology, 06 April 2004, Vol. 174, No. 8, pg 5024-5032; pg 5024, col 2, para 1; pg 5025, col 2, para 1; pg 5026, col 2, para 1; pg 5026, col 2, para 3; pg 5027, col 1, para 2; pg 5027, col 2, para 3; pg 5027, col 2, para 4; pg 5028, col 2, para 2	1-2, 6, 9-16 ---------- 3-5, 7-8
Y	DURHAM et al. Expression of E germ-line gene transcripts and mRNA for the E heavy chain of IgE in nasal B cells and the effects of topical corticosteroid. European Journal of Immunology, November 1997, Vol. 27, No. 11, pg 2899-2906; pg 2902, col 1, para 2; pg 2902, col 2, para 1; pg 2903, col 2, para 1	3-5
Y	CROOTE et al. Human IgE producing B cells have a unique transcriptional program and generate high affinity, allergen-specific antibodies. BioRxiv, 22 May 2018, [online]. [Retrieved on 17 May 2022]. Retrieved from the internet: <URL: https://www.biorxiv.org/content/10.1101/327866v1.full.pdf> Especially pg 5, ln 182-201	7-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search	Date of mailing of the international search report
13 May 2022	JUN 16 2022
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