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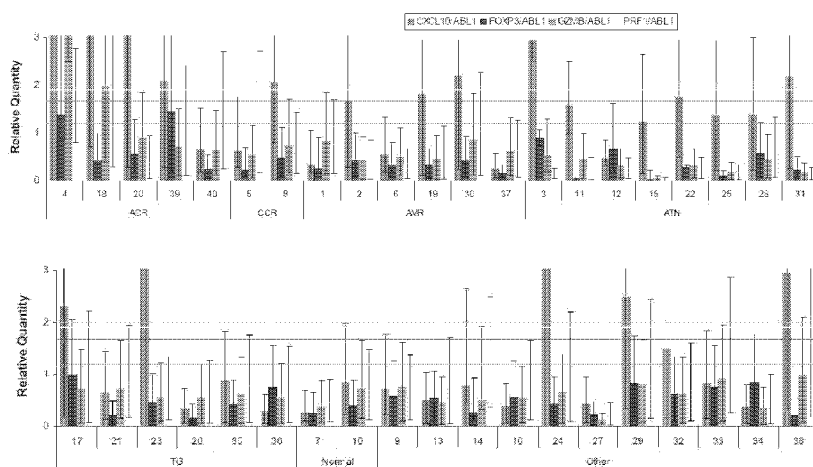
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(54) **Title:** MOLECULAR MARKERS FOR DIAGNOSIS OF ACUTE CELLULAR REJECTION AND ACUTE TUBULAR NECROSIS**FIG. 1A**

(57) **Abstract:** Provided herein are methods for identifying a renal transplant patient as likely suffering renal graft rejection, comprising determining the expression level of one or more genes selected from the group consisting of ABL1, NK1, NK3, GZMB, PRF1, FDXP3 and CXCL10 in a sample isolated from a patient and identifying the patient as likely suffering renal graft rejection if at least one of the one or more genes is overexpressed in the sample. Once a patient that suffers renal graft rejection is identified, the disclosure also provides methods for treating the rejection.

Molecular Markers for Diagnosis of Acute Cellular Rejection and Acute Tubular Necrosis

TECHNICAL FIELD

[0001] The present disclosure generally relates to methods for diagnosis of renal graft rejections including, in particular, acute cellular rejection (ACR), acute tubular necrosis (ATN), in a renal transplant recipient. Once the rejection is detected, methods for treating the rejection are provided.

BACKGROUND

[0002] The following description is provided to assist the understanding of the reader. None of the information provided or references cited is admitted to be prior art.

[0003] Renal transplant recipients may suffer graft rejections, such as acute cellular rejection (ACR), acute tubular necrosis (ATN), antibody mediated rejection (ABMR), and other types of rejection reactions. T-cells, part of the adaptive immune system, play a role in ACR. Natural killer cells (NKC) of the innate immune system are known to play a role in ATN and ABMR.

[0004] Renal graft rejections, including ACR and ATN, are usually diagnosed on the basis of histologic evaluation performed in response to biochemical evidence of graft impairment (*e.g.*, elevated creatinine levels). There is a need to develop more accurate and noninvasive tests for renal graft rejections that may allow earlier detection and intervention.

SUMMARY

[0005] The present disclosure provides a method for identifying a renal transplant patient as likely suffering renal graft rejection, comprising: determining the expression level of one or more genes selected from the group consisting of c-abl oncogene 1 non-receptor tyrosine kinase (ABL1), CD160 molecule (NK1 or CD160), natural cytotoxicity triggering receptor 3 (NK3 or NCR3), granzyme B (GZMB), perforin 1 (PRF1), forkhead box P3 (FOXP3) and chemokine (C-X-C motif) ligand 10 (CXCL10) in a sample isolated from a patient; and identifying the patient as likely suffering renal graft rejection if at least one of the one or more genes is overexpressed in the sample. The renal graft rejection comprises one or more conditions selected from the group consisting of acute cellular rejection (ACR), acute tubular

necrosis (ATN), antibody mediated rejection (ABMR), chronic cellular rejection, borderline rejection, diabetic nephropathy, transplant nephropathy, and minimal interstitial fibrosis or tubular atrophy. According to the methods described herein, the patient is identified as likely suffering ATN or ACR if at least one of CXCL10, FOXP3, GZMB or PRF1 is overexpressed in the sample, and in particular, is identified as likely suffering ATN or ACR if at least two of CXCL10, FOXP3, GZMB or PRF1 are overexpressed in the sample. Furthermore, the patient is identified as likely suffering ATN if (a) ABL1 and (b) NK1 or NK3 are overexpressed in the sample, and the patient is identified as likely suffering ACR if none of ABL1, NK1 and NK3 is overexpressed in the sample. Overexpression is determined as an increase of expression level above a reference range of expression level of the corresponding gene in a normal population, and the reference ranges are provided in Table 1.

BRIEF DESCRIPTION OF THE FIGURES

[0006] FIG. 1A-B show transcription levels of examined genes (GZMB, PRF1, FOXP3 and CXCL10) relative to ABL1 (**FIG. 1A**) and CD3e (**FIG. 1B**) in renal transplant recipients. Patient classification is based on renal histology. “Other” includes chronic cellular rejection, borderline rejection, diabetic nephropathy, transplant nephropathy, and minimal interstitial fibrosis or tubular atrophy. X-axis shows patient numbers. Error bars are 95% CI based on 4 replicates. Bars without errors bars exceed the Y-axis range. Reference ranges are in **Table 1**.

[0007] FIG. 2A-B show transcription levels of natural killer genes (NK1 and NK2) relative to ABL1 (**FIG. 2A**) and CD3e (**FIG. 2B**) in renal transplant recipients. Classification is based on renal histology. “Other” includes chronic cellular rejection, borderline rejection, diabetic nephropathy, transplant nephropathy, and minimal interstitial fibrosis or tubular atrophy. X-axis shows patient numbers. Error bars are 95% CI based on 4 replicates. Bars without errors bars exceed the Y-axis range. Reference ranges are in **Table 1**.

[0008] FIG. 3A-M present the relative gene expression quantities of the marker genes (as indicated) for a patient, S01, that did not experience renal graft rejections. The x-axis indicates days following the renal transplant. The dotted lines show the calculated elevation thresholds for each gene. **FIG. 3A-F** use Abl1 for normalization (as indicated) and **FIG. 3G-M** use CD3d for normalization (as indicated).

[0009] **FIG. 4A-W** present relative gene expression quantities of the marker genes (as indicated) for a patient, R01, that experienced acute cellular rejection (ACR). The x-axis indicates days following the renal transplant. The dotted lines show the calculated elevation thresholds for each gene. **FIG. 4A-J** use Abl1 for normalization (as indicated) and **FIG. 4K-W** use CD3d for normalization (as indicated). The gene expression levels for some of the genes are twice, at different scales for clarity (see, e.g., **FIG. 4A-B**).

DETAILED DESCRIPTION

[0010] The present disclosures are based on the identification of gene expression markers useful for detection of renal graft rejections in a renal transplant patient.

[0011] As used in this specification and the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the content clearly dictates otherwise. For example, reference to “a nucleic acid” includes a combination of two or more nucleic acids, and the like.

[0012] As used herein, “about” will be understood by persons of ordinary skill in the art and will vary to some extent depending upon the context in which it is used. For example, when referring to a relative quantity of a gene, “about” refers to ± 0.1 fold.

[0013] One embodiment of the present disclosure provides a method for identifying a renal transplant patient as likely suffering renal graft rejection. The method entails determining the expression level of one or more genes selected from the group consisting of c-abl oncogene 1 non-receptor tyrosine kinase (ABL1), CD160 molecule (NK1 or CD160), natural cytotoxicity triggering receptor 3 (NK3), granzyme B (GZMB), perforin 1 (PRF1), forkhead box P3 (FOXP3) and chemokine (C-X-C motif) ligand 10 (CXCL10 or IP10) in a sample isolated from a patient.

[0014] As the experimental example shows, the expression levels of all of the genes are generally within a “reference range” in samples isolated from patients not suffering renal graft rejections (“normal” patients). The majority of patients that suffer from one or more of such rejections, by contrast, exhibited “overexpression” of one or more of these genes. Accordingly, overexpression of one or more these genes can aid in the diagnosis of these rejections.

[0015] The expression level of a gene in a sample, in one aspect, is expressed as a “relative quantity” or “RQ” number, which is the ratio between the measured expression of the gene in a test sample and the measured expression of the gene in a control sample. In some aspects, the control sample is a sample isolated from a patient that does not suffer renal graft rejections. In some aspects, the control sample is a sample prepared by pooling two or more such control samples. In yet another aspect, a virtual control sample can be prepared by mathematically summarizing the expression of the gene in more than one control samples.

[0016] A gene is “overexpressed” in a test sample when the relative quantity of the gene in the test sample is above a “reference range.” As used herein, a “reference range” reflects a normal range of relative quantities that a gene exhibits in control samples. A gene’s reference range can be determined by measuring the expression levels of the gene in two, or three or more control samples, following statistical summarization of the expression levels. For instance, Gaussian distribution can be used to calculate the reference ranges.

[0017] In some aspects, before the relative quantity of a gene in a sample is calculated, the raw gene expression value can be normalized against a control gene. In some aspects, the control gene is a housekeeping gene. In one aspect, the control gene is ABL1, and in another aspect, the control gene is CD3e molecule epsilon (CD3e). Exemplary reference ranges for genes in the present disclosure are provided in **Table 1**.

[0018] “Renal graft rejections” refer to rejections of a transplanted renal tissue by the recipient’s immune system, which cause injury in or destroy the transplanted renal tissue. Various types of renal graft rejections have been observed clinically, including without limitation acute cellular rejection (ACR), acute tubular necrosis (ATN), antibody mediated rejection (ABMR), chronic cellular rejection, borderline rejection, diabetic nephropathy, transplant nephropathy, and minimal interstitial fibrosis or tubular atrophy. Methods for histopathological identification of these rejections are known in the art. See, for instance, Mauiyyedi and Colvin, *Current Opinion in Nephrology & Hypertension*, **11**(6):609-18 (2002).

[0019] It has been observed that, when at least one of the genes is overexpressed in the sample, the patient from which the sample is isolated can be identified as likely suffering from one or more of renal graft rejections. This is based on the fact that none of the normal patients exhibited overexpression of any of these genes, and only those that suffer from the

rejection did. In particular, as the experimental example demonstrates, when at least one of GZMB, PRF1, FOXP3 or CXCL10 is overexpressed, the patient likely suffers ATN or ACR. In another embodiment, when at least two, or alternatively at least three, or all four of GZMB, PRF1, FOXP3 or CXCL10 are overexpressed, the patient likely suffers ATN or ACR.

[0020] The present disclosure further provides markers for distinguishing ATN from ACR. It has been discovered, unexpectedly, that ABL1, a putative housekeeping gene, was overexpressed in all ATN patients. Furthermore, many ATN patients exhibited overexpression of either or both of NK1 and NK3. In one embodiment of the method, therefore, the patient is identified as likely suffering ATN if (a) ABL1 and (b) NK1 or NK3 are overexpressed in the sample.

[0021] In another embodiment, the patient is identified as likely suffering ACR if none of ABL1, NK1 and NK3 is overexpressed in the sample.

[0022] It is further observed that the overexpression of one or more of these marker genes happens before the rise of the creatinine level, which is a conventional clinical marker for renal graft rejection. Accordingly, this shows that the prediction of renal graft rejection with the present gene markers can be made even before what can be done with the conventional clinical methods.

[0023] In some embodiments, therefore, the method of the present disclosure entails the detection of the gene expression levels of the genes in sample that is isolated before the rise of the creatinine level in a patient, or at a time that is prior to the anticipated rise of the creatinine level. Accordingly, provided in one embodiment is a method for identifying a renal transplant patient as likely suffering renal graft rejection, comprising determining the expression level of one or more genes selected from the group consisting of c-abl oncogene 1 non-receptor tyrosine kinase (ABL1), CD160 molecule (NK1), natural cytotoxicity triggering receptor 3 (NK3), granzyme B (GZMB), perforin 1 (PRF1), forkhead box P3 (FOXP3) and chemokine (C-X-C motif) ligand 10 (CXCL10) in a sample isolated from a patient that has undergone renal transplant and has a level of creatinine within a normal physiological range; and identifying the patient as likely suffering renal graft rejection if at least one, or two, or three, or four of the one or more genes are overexpressed in the sample.

[0024] Normal physiological ranges are known the art. For instance, normal levels of creatinine in the blood are approximately 0.6 to 1.2 milligrams (mg) per deciliter (dL) in adult males and 0.5 to 1.1 milligrams per deciliter in adult females. Therefore, in some embodiments, the patient undergoing the detection has a blood creatinine level that is equal to or lower than about 1.0 milligrams per deciliter, 1.1 milligrams per deciliter, or 1.2 milligrams per deciliter, or 1.3 milligrams per deciliter, or 1.4 milligrams per deciliter, or 1.5 milligrams per deciliter, or 2 milligrams per deciliter.

Sample Processing and Expression Level Determination

[0025] In one embodiment, the expression levels of one or more genes are determined in a sample isolated from a renal transplant patient.

[0026] As used herein, the term “sample” or “test sample” refers to any liquid or solid material containing nucleic acids. In one embodiment, a sample is a blood or tissue sample from a human patient. In another embodiment, a sample comprises peripheral blood or plasma.

[0027] In some aspects, the expression level of a gene refers to the mRNA expression level of the gene. Various methods of nucleic acid extraction are suitable for isolating total RNA or mRNA. See Maniatis et al., *Molecular Cloning, A Laboratory Manual*, 2d, Cold Spring Harbor Laboratory Press, page 16.54 (1989). Numerous commercial kits also yield suitable RNA including, but not limited to, QIAamp™ mini blood kit, Agencourt Genfind™, Roche Cobas® Roche MagNA Pure® or phenol:chloroform extraction using Eppendorf Phase Lock Gels®, and the NucliSens extraction kit (Biomerieux, Marcy l'Etoile, France). In other methods, mRNA may be extracted from patient blood/bone marrow samples using MagNA Pure LC mRNA HS kit and Mag NA Pure LC Instrument (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN).

[0028] Nucleic acid extracted from tissues, cells, plasma or serum can be amplified using nucleic acid amplification techniques well known in the art. Many of these amplification methods can also be used to detect the presence of mutations simply by designing oligonucleotide primers or probes to interact with or hybridize to a particular target sequence in a specific manner. By way of example, but not by way of limitation these techniques can include the polymerase chain reaction (PCR) reverse transcriptase polymerase chain reaction

(RT-PCR), nested PCR, ligase chain reaction. See Abravaya, K., et al., *Nucleic Acids Research* 23:675-682, (1995), branched DNA signal amplification, Urdea, M. S., et al., *AIDS* 7 (suppl 2):S11-S 14, (1993), amplifiable RNA reporters, Q-beta replication, transcription-based amplification, boomerang DNA amplification, strand displacement activation, cycling probe technology, isothermal nucleic acid sequence based amplification (NASBA). See Kievits, T. et al., *J Virological Methods* 35:273-286, (1991), Invader Technology, or other sequence replication assays or signal amplification assays.

[0029] Some methods employ reverse transcription of RNA to cDNA. As noted, the method of reverse transcription and amplification may be performed by previously published or recommended procedures, which referenced publications are incorporated herein by reference in their entirety. Various reverse transcriptases may be used, including, but not limited to, MMLV RT, RNase H mutants of MMLV RT such as Superscript and Superscript II (Life Technologies, GIBCO BRL, Gaithersburg, Md.), AMV RT, and thermostable reverse transcriptase from *Thermus thermophilus*. For example, one method, but not the only method, which may be used to convert RNA extracted from plasma or serum to cDNA is the protocol adapted from the Superscript II Preamplification system (Life Technologies, GIBCO BRL, Gaithersburg, Md.; catalog no. 18089-011), as described by Rashtchian, A., *PCR Methods Applic.* 4:S83-S91, (1994), adapted as follows.

[0030] For instance, one to five micrograms of RNA extracted from plasma or serum in 13 μ l of DEPC-treated water is added to a clean microcentrifuge tube. Then one microliter of either oligo (dT) (0.5 mg/ml) or random hexamer solution (50 ng/ μ l) is added and mixed gently. The mixture is then heated to 70 degrees centigrade for 10 minutes and then incubated on ice for one minute. Then, it is centrifuged briefly followed by the addition of 2 μ l of 10 $\times$ synthesis buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 25 mM magnesium chloride, 1 mg/ml of BSA), 1 μ l of 10 mM each of dNTP mix, 2 μ l of 0.1 M DTT, 1 μ l of SuperScript II RT (200 U/ μ l) (Life Technologies, GIBCO BRL, Gaithersburg, Md.). After gentle mixing, the reaction is collected by brief centrifugation, and incubated at room temperature for 10 minutes. The tube is then transferred to a 42°C water bath or heat block and incubated for 50 minutes. The reaction is then terminated by incubating the tube at 70°C for 15 minutes, and then placing it on ice. The reaction is collected by brief centrifugation, and 1 μ l of RNase H (2 units) is added followed by incubation at 37°C for 20 minutes before proceeding to nucleic acid amplification.

[0031] To the cDNA mixture add the following: 8 μ l of 10 $\times$ synthesis buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 25 mM magnesium chloride, 1 mg/ml of BSA), 68 μ l sterile double-distilled water, 1 μ l amplification primer 1 (10 μ M), 1 μ l amplification primer 2 (10 μ M), 1 μ l Taq DNA polymerase (2-5 U/ μ l). Mix gently and overlay the reaction mixture with mineral oil. The mixture is heated to 94°C for 5 minutes to denature remaining RNA/cDNA hybrids. PCR amplification is then performed in an automated thermal-cycler for 15-50 cycles, at 94°C for 1 minute, 55° for 30 to 90 seconds, and 72°C for 2 minutes.

[0032] Cycling parameters and magnesium concentration may vary depending upon the specific sequence to be amplified, however, optimization procedures and methods are also well known in the art.

[0033] Nucleic acid sequences of the genes useful for identifying renal transplant patients that suffer renal graft rejections are known in the art, and examples are provided in the following table.

Gene Symbol	Gene Name	Exemplary GenBank Accession No. for mRNA Sequence
ABL1	c-abl oncogene 1 non-receptor tyrosine kinase	NM_005157
NK1	CD160 molecule	NM_007053
NK3	natural cytotoxicity triggering receptor 3	NM_147130
GZMB	granzyme B	NM_004131
PRF1	perforin 1	NM_001083116
FOXP3	forkhead box P3	NM_014009
CXCL10 or IP10	chemokine (C-X-C motif) ligand 10	NM_001565
CD3e	CD3e molecule epsilon	NM_000733

Treatment of Identified Patients

[0034] Once a renal transplant patient is identified as likely suffering from renal graft rejection, the present disclosure further provides methods for treating the rejection.

[0035] As used herein, the terms “treating” or “treatment” or “alleviation” refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. A subject is successfully “treated” for a disorder if, after receiving a therapeutic agent according to the methods of the present disclosure, the subject shows observable and/or measurable reduction in or absence of one or more signs and symptoms of a particular disease or condition.

[0036] Methods for treating renal graft rejections are known in the art. Acute tubular necrosis (ATN), for instance, can be treated with dialysis or plasma exchange. See, *e.g.*, Esson and Schrler, *Ann. Intern. Med.* 137:744-52 (2002).

[0037] Treatment options for acute cellular rejection (ACR), likewise, are also known. For instance, corticosteroids are typically recommended as the initial treatment for ACR. Additionally, for those that do not respond to corticosteroids, lymphocyte-depleting antibodies or OKT3 can be administered. Plasma exchange can also be useful for treating or alleviating symptoms of ACR.

EXPERIMENTAL EXAMPLES

[0038] The present disclosure is further illustrated by the following examples, which should not be construed as limiting in any way.

Example 1

[0039] This example investigated differential expression patterns of several genes in patients with various forms of renal graft failure. These genes included PRF1, GZMB, FOXP3, and CXCL10 (IP10). Initial experiments used CD3e and ABL1 (a housekeeping gene) as internal controls for relative quantitation.

[0040] During the initial study, however, this example observed aberrant levels of ABL1 expression (relative to CD3e) in samples from ATN patients. Since natural killer (NK) cells produce ABL1 but not CD3e, this example also investigated whether two known markers of NK cells, NK1 and NK3, might help further differentiate ATN from ACR.

Methods and Materials:

[0041] *Patients:* 40 renal transplant recipients undergoing renal biopsy for cause (elevated creatinine). **FIG. 1-2** show the distribution of histology. One hundred twenty apparently healthy, ambulatory, community-dwelling adults were recruited to establish reference ranges. Individuals with a known history of renal, autoimmune, or inflammatory disease were excluded.

[0042] *Molecular Detection Assays:* RNA was extracted from peripheral blood in PAXgene® tubes (QIAGEN Inc, Valencia, CA) using the Roche MagNA Pure method

(Roche Applied Science, Indianapolis, IN). Extracted RNA was then reverse-transcribed to generate cDNA for real-time PCR on the ABI Viia® 7 thermal cycler (Applied Biosystems, Foster City, CA).

[0043] Targeted gene expression markers included two cytotoxic T lymphocyte markers (GZMB; PRF1), the regulatory T lymphocyte transcription factor FOXP3 (FOXP3), a chemokine expressed in T lymphocytes and monocytes (IP10, CXCL10); and the NK cell markers NK1 and NK3. The T lymphocyte marker CD3e and the pan-cell marker ABL1 were used as constitutively expressed endogenous normalizer genes. Use of automated RNA processing and multi-sample automated cDNA amplification allowed high throughput of sample sets.

[0044] Each target was amplified in 4 replicate reactions. Cycle threshold (Ct) values were compared to a normal pooled control sample via the delta-delta-Ct method to give a relative quantity (RQ) for each transcript (normalized to one of the marker genes). Markers with RQ values above the reference range values were considered elevated.

Results:

[0045] Reference range studies are summarized in Table 1. Values for all markers followed a Gaussian distribution with either logarithmic or square-root transformation.

Table 1. Reference Ranges for Marker Ratios (Relative Quantity, RQ)

Marker	Reference Range	
	Normalized to ABL1	Normalized to CD3e
GZMB	0.3-2.0	0.3-2.4
PRF1	0.4-1.9	0.4-2.2
FOXP3	0.5-1.7	0.5-1.8
IP10	0.1-1.2	0.1-1.1
NK1	0.4-1.9	0.4-2.5
NK3	0.5-1.8	0.5-2.1

[0046] Results grouped by renal histology findings are summarized in **FIG. 1 and 2**. ACR and ATN patients showed marked elevations in several markers, with differential patterns of overexpression.

[0047] For ACR, when the expression levels of the genes were evaluated using ABL1 as control: 4 of 5 ACR patients showed markedly elevated CXCL10; 1 showed elevated GZMB; and 1 had slightly elevated PRF1. One ACR patient had elevated NK3, but all other ACR

patients had normal levels of both NK markers. When using CD3e as control: 3 of 5 ACR patients showed markedly elevated CXCL10; 1 had elevated GZMB; 1 had slightly elevated PRF1; 1 had elevated ABL1; and none had elevated FOXP3.

[0048] With respect to ATN, when the gene expression levels of the genes were evaluated relative to ABL1: All 8 ATN patients had at least mildly elevated CXCL10 levels; no other markers were elevated, including NK1 and NK3. Using CD3e as reference: All 8 ATN patients showed elevations ABL1 as well as 3 or 4 of the ACR markers (CXCL10, FOXP3, GZMB, PRF1). NK1 was elevated in 7 patients, and NK3 in 6 patients.

[0049] Few transplant recipients with other causes of rejection showed marker elevations, and most of these elevations were mild (see **FIG. 1 and 2**).

[0050] These findings demonstrate that constitutively expressed NK cell mRNAs for NK1 and NK3 have potential value as markers of ATN in renal transplant recipients. Further, information from markers of ACR (CXCL10, FOXP3, GZMB, PRF1) and NK cells (NK1, NK3) may help distinguish between ACR and ATN. Both ATN and ACR may be associated with elevated levels of more than one ACR markers. In this context, ATN is characterized by elevated ABL1 plus NK1 or NK3, along with more than one ACR marker, and ACR is generally characterized by normal levels of ABL1, NK1, and NK3.

Example 2

[0051] The data presented in this example were collected from a pilot human study which included 10 renal transplant recipients. Out of the 10 renal transplant recipients, seven did not exhibit clinical signs of rejection and were considered “stable”. Three of the recipients had ACR, one of which had an episode of ATN.

[0052] Gene expressions of the panel of markers identified in Example 1, including PRF1, GZMB, FOXP3, CXCL10 (IP10), NK1, NK3, CD3e and ABL1, were tested in blood samples collected from these patients. The gene expression data were then evaluated according to the criteria generated in Example 1.

[0053] All seven stable renal transplant recipients did not meet the ACR criteria of Example 1. **FIG. 3A-M** present the relative quantity of the genes measured from one of the stable patients, R01. When normalized with Abl1, the marker genes, CXCL10 (**FIG. 3A**),

FOXP3 (**FIG. 3B**), GZMB (**FIG. 3C**), NK1 (**FIG. 3D**), NK3 (**FIG. 3E**) and PRF1 (**FIG. 3F**), showed relative expression levels below the thresholds (indicated by dotted horizontal lines above the x-axis) on each day post-transplantation (indicated on the x-axis) when samples were collected, until 336 days. Likewise, when normalized with CD3e, none of these genes, plus Abl1, showed elevated expression (**FIG. 3G-M**).

[0054] Two of the three ACR patients met the ACR criteria. The gene expression data from one of the two ACR patients, R01, are presented in **FIG. 4A-W**. When normalized with Abl1, CXCL10 (**FIG. 4A-B**), GZMB (**FIG. 4D-E**), NK1 (**FIG. 4F-G**), PRF1 (**FIG. 4I-J**) showed elevated expression at as early as 5 days post-transplantation (see **FIG. 4A-B**). The expression elevation of NK3 (**FIG. 4H**) was relative late, observed first on the 336th day (**FIG. 4H**). Only FOXP3 (**FIG. 4C**) did not show elevation. Similar elevation of gene expression levels is shown in **FIG. 4K-V**, when normalized with CD3e. Again, only FOXP3 (**FIG. 4M**) did not show elevation and the elevation for other genes were seen at as early as 7 weeks post-transplantation (**FIG. 4K-L**). The elevation of these gene markers, therefore, are valid markers predicting the renal rejections. It was also observed that the elevation started even before the rise of creatinine, a conventional clinical marker for renal rejections.

[0055] The remaining ACR patient did not meet the ACR criteria with respect to expression levels of the marker genes. This patient was suspected to have undergone heavy immunosuppression therapy. Nevertheless, even without confirming the medication history of this patient, the prediction accuracy of the gene markers were 90%, with 100% specificity (all stable patients were correctly predicted) and 67% sensitivity (two of three rejected patients correctly predicted).

[0056] The results of this example therefore confirms that the gene expression levels of PRF1, GZMB, FOXP3, CXCL10 (IP10), NK1, NK3, CD3e and ABL1 are valid markers for renal graft rejections.

* * * *

[0057] The present disclosure is not to be limited in terms of the particular embodiments described in this application. Many modifications and variations can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatuses within the scope of the disclosure, in addition to those enumerated herein, will be apparent to those skilled in the art from the

foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. The present disclosure is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled. It is to be understood that this disclosure is not limited to particular methods, reagents, compounds compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0058] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0059] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language including, but not limited to, *e.g.*, “up to,” “at least,” “greater than,” “less than,” and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member.

[0060] While various aspects and embodiments have been disclosed herein, other aspects and embodiments will be apparent to those skilled in the art. The various aspects and embodiments disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

CLAIMS:

1. A method for identifying a renal transplant patient as likely suffering renal graft rejection, comprising:
determining the expression level of one or more genes selected from the group consisting of c-abl oncogene 1 non-receptor tyrosine kinase (ABL1), CD160 molecule (NK1), natural cytotoxicity triggering receptor 3 (NK3), granzyme B (GZMB), perforin 1 (PRF1), forkhead box P3 (FOXP3) and chemokine (C-X-C motif) ligand 10 (CXCL10) in a sample isolated from a patient that has undergone renal transplant; and
identifying the patient as likely suffering renal graft rejection if at least one of the one or more genes is overexpressed in the sample.
2. The method of claim 1, wherein the renal graft rejection comprises one or more conditions selected from the group consisting of acute cellular rejection (ACR), acute tubular necrosis (ATN), antibody mediated rejection (ABMR), chronic cellular rejection, borderline rejection, diabetic nephropathy, transplant nephropathy, and minimal interstitial fibrosis or tubular atrophy.
3. The method of claim 1, wherein the patient is identified as likely suffering ATN or ACR if at least one of CXCL10, FOXP3, GZMB or PRF1 is overexpressed in the sample.
4. The method of claim 3, wherein the patient is identified as likely suffering ATN or ACR if at least two of CXCL10, FOXP3, GZMB or PRF1 are overexpressed in the sample.
5. The method of claim 3 or 4, wherein the patient is identified as likely suffering ATN if (a) ABL1 and (b) NK1 or NK3 are overexpressed in the sample.
6. The method of claim 3 or 4, wherein the patient is identified as likely suffering ACR if none of ABL1, NK1 and NK3 is overexpressed in the sample.

7. The method of any one of claims 1-6, wherein overexpression is determined as compared to the expression level of the corresponding gene in a corresponding sample from a patient that does not experience renal graft rejection.
8. The method of claim 7, wherein the comparison is normalized using a control gene.
9. The method of claim 8, wherein the control gene is CD3e molecule epsilon (CD3e).
10. The method of claim 9, wherein overexpression is determined as an increase of expression level above a reference range of expression level of the corresponding gene in a normal population.
11. The method of claim 10, wherein the reference ranges are provided in Table 1.
12. The method of any preceding claim, wherein the sample is isolated before the level of creatinine is elevated in the patient.
13. The method of any preceding claim, wherein the sample comprises peripheral blood.
14. The method of any preceding claim, wherein the expression levels are determined using real time PCR.
15. The method of any preceding claim, further comprising treating the renal graft rejection.
16. The method of claim 15, wherein the treatment comprises one or more of dialysis, plasma exchange, administration of a corticosteroid, administration of a lymphocyte-depleting antibody, or administration of mycophenolate.
17. A method for identifying a renal transplant patient as likely suffering renal graft rejection, comprising:
determining the expression level of one or more genes selected from the group consisting of c-abl oncogene 1 non-receptor tyrosine kinase (ABL1), CD160 molecule (NK1), natural cytotoxicity triggering receptor 3 (NK3), granzyme B

(GZMB), perforin 1 (PRF1), forkhead box P3 (FOXP3) and chemokine (C-X-C motif) ligand 10 (CXCL10) in a sample isolated from a patient that has undergone renal transplant and has a blood creatinine level within a normal physiological range; and
identifying the patient as likely suffering renal graft rejection if at least one of the one or more genes is overexpressed in the sample.

18. The method of claim 17, wherein patient has a blood creatinine level that is equal to or lower than about 1.2 milligrams per deciliter.
19. The method of claim 17, further comprising treating the renal graft rejection.
20. The method of claim 19, wherein the treatment comprises one or more of dialysis, plasma exchange, administration of a corticosteroid, administration of an lymphocyte-depleting antibody, or administration of mycophenolate.

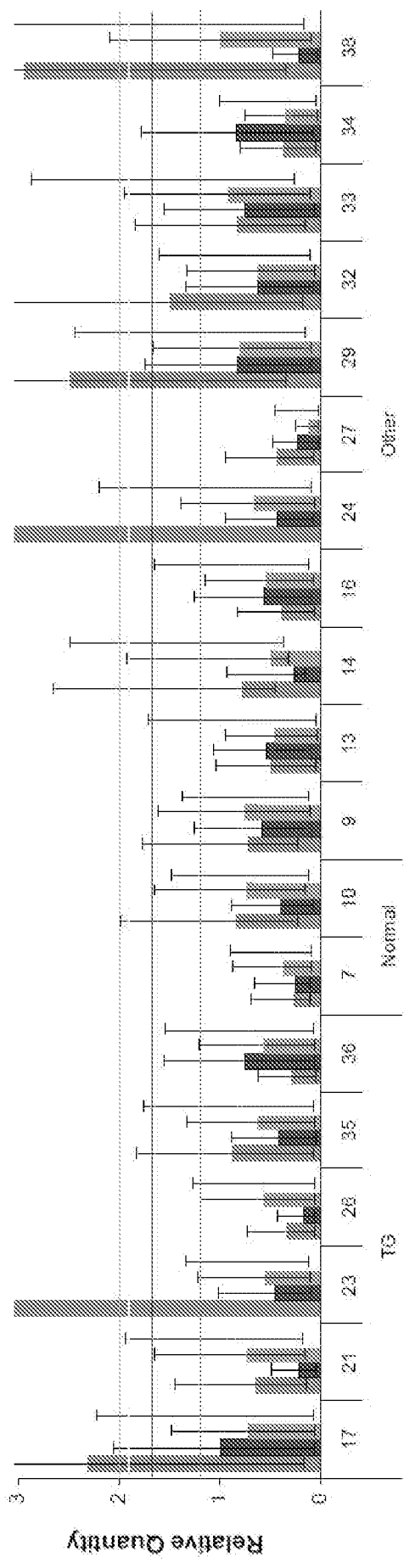
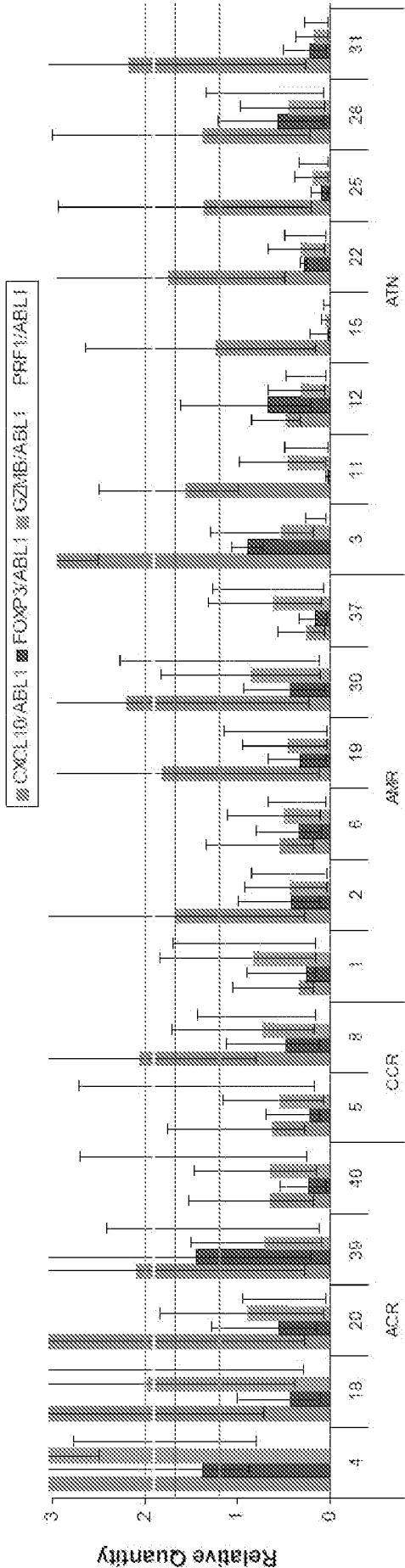


FIG. 1A

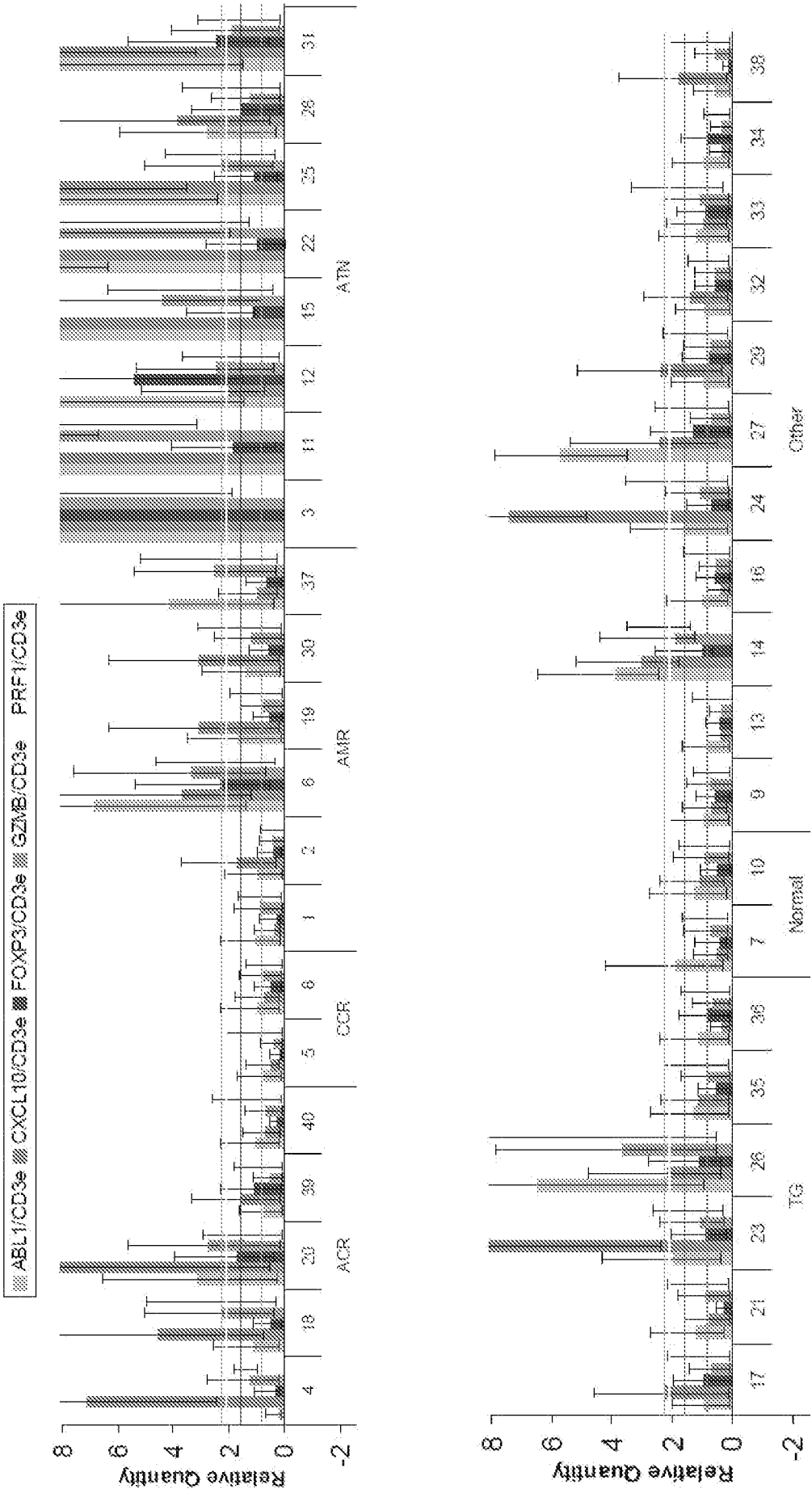


FIG. 1B

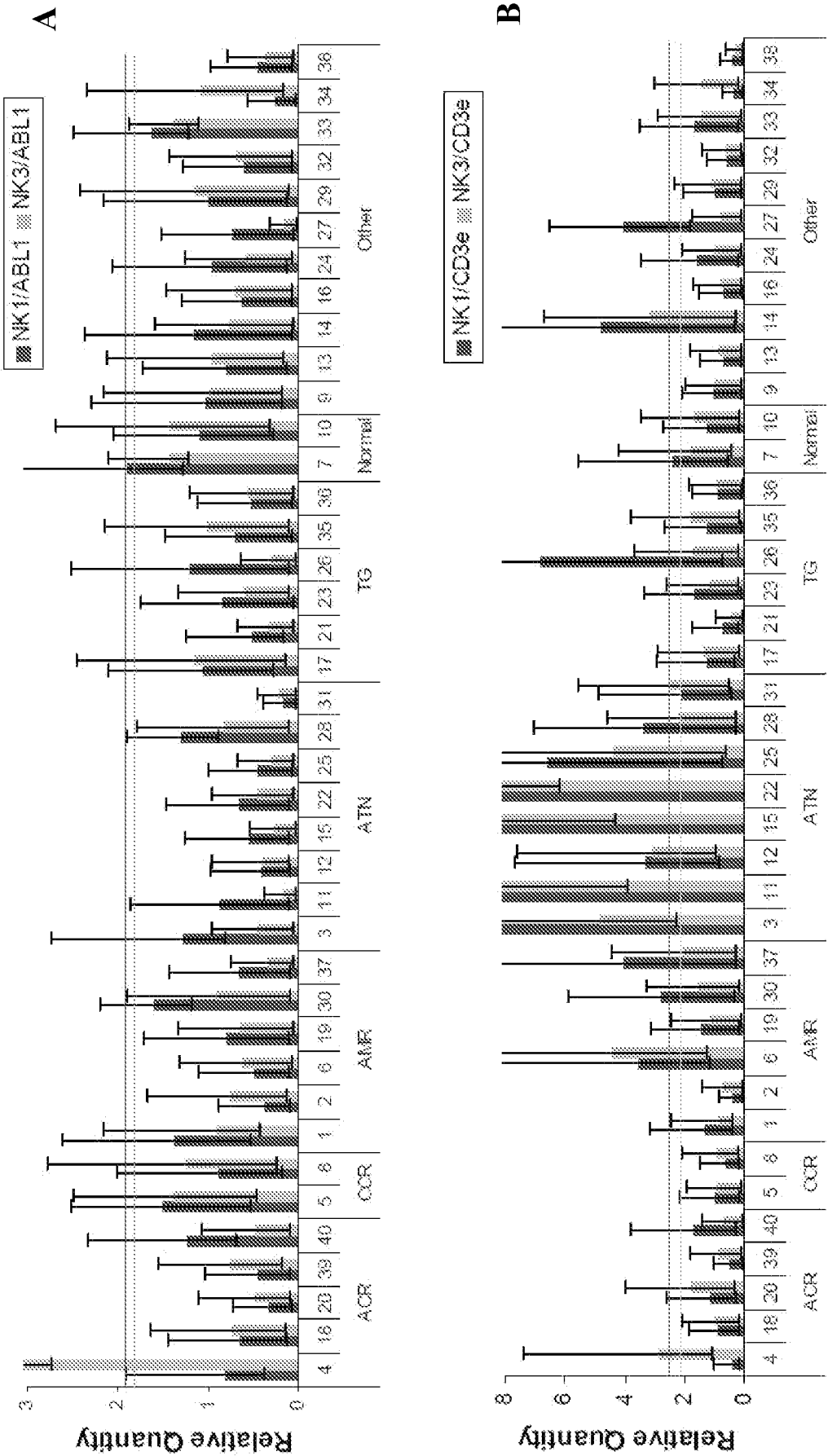


FIG. 2

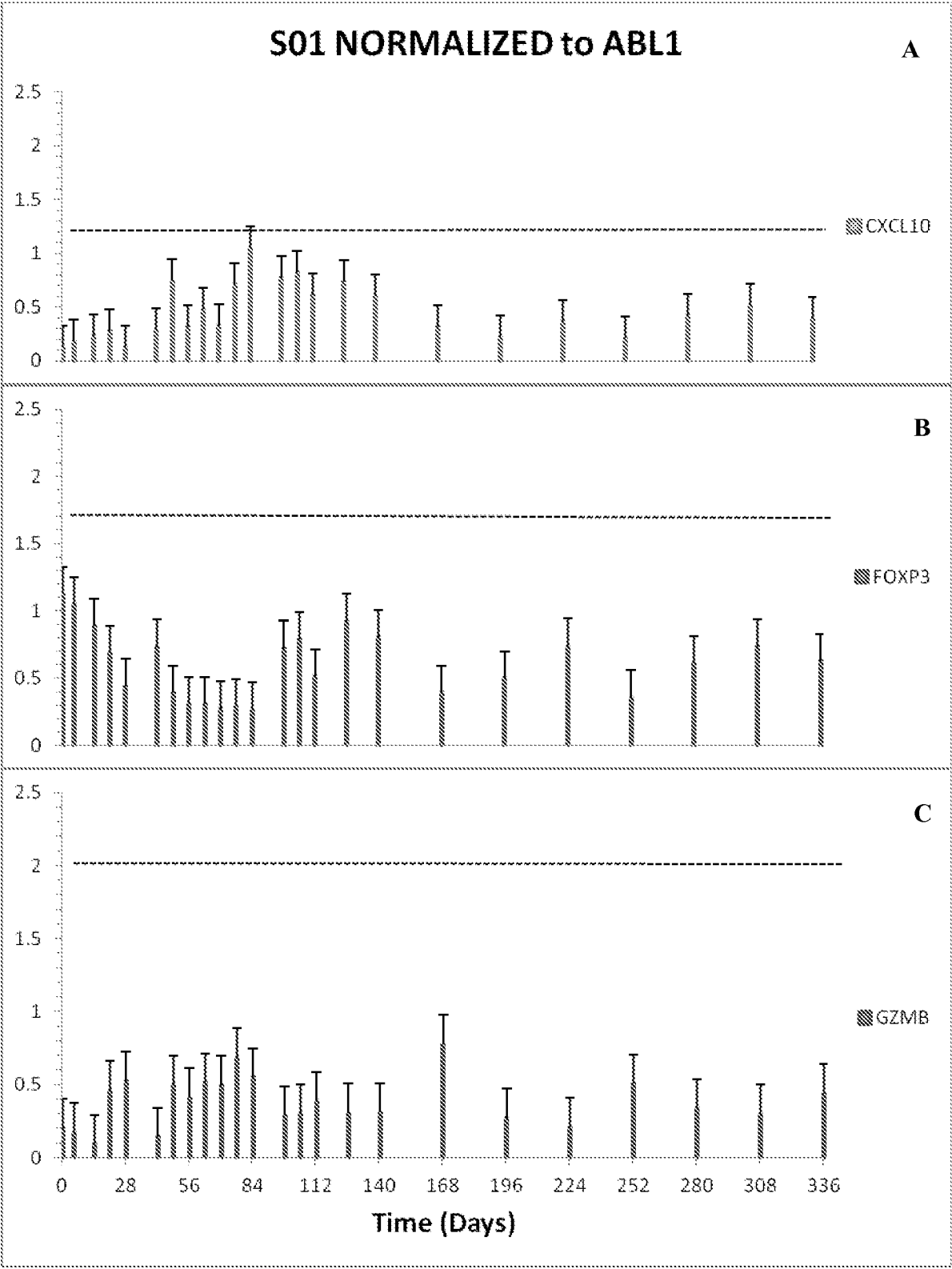


FIG. 3A-C

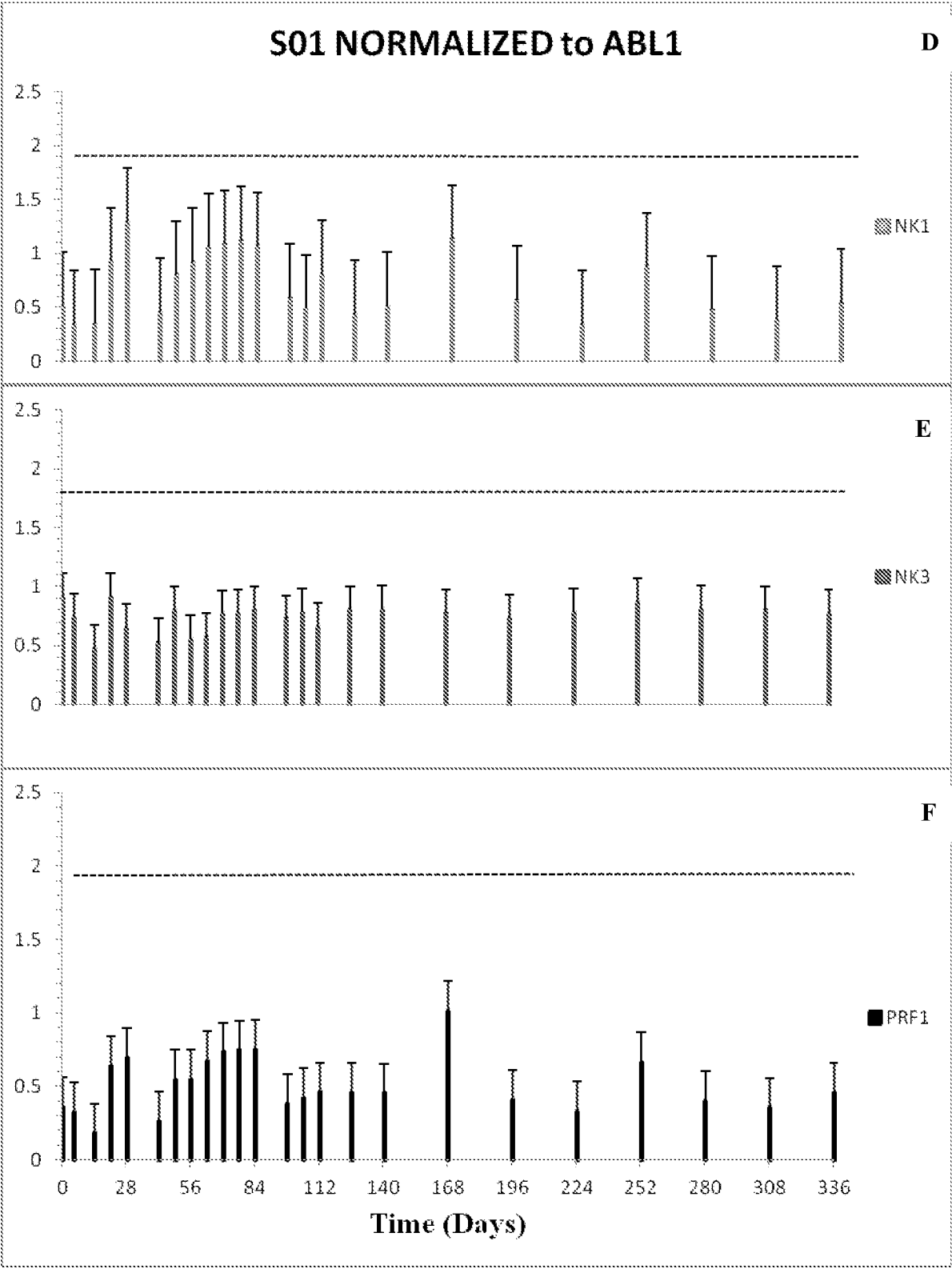
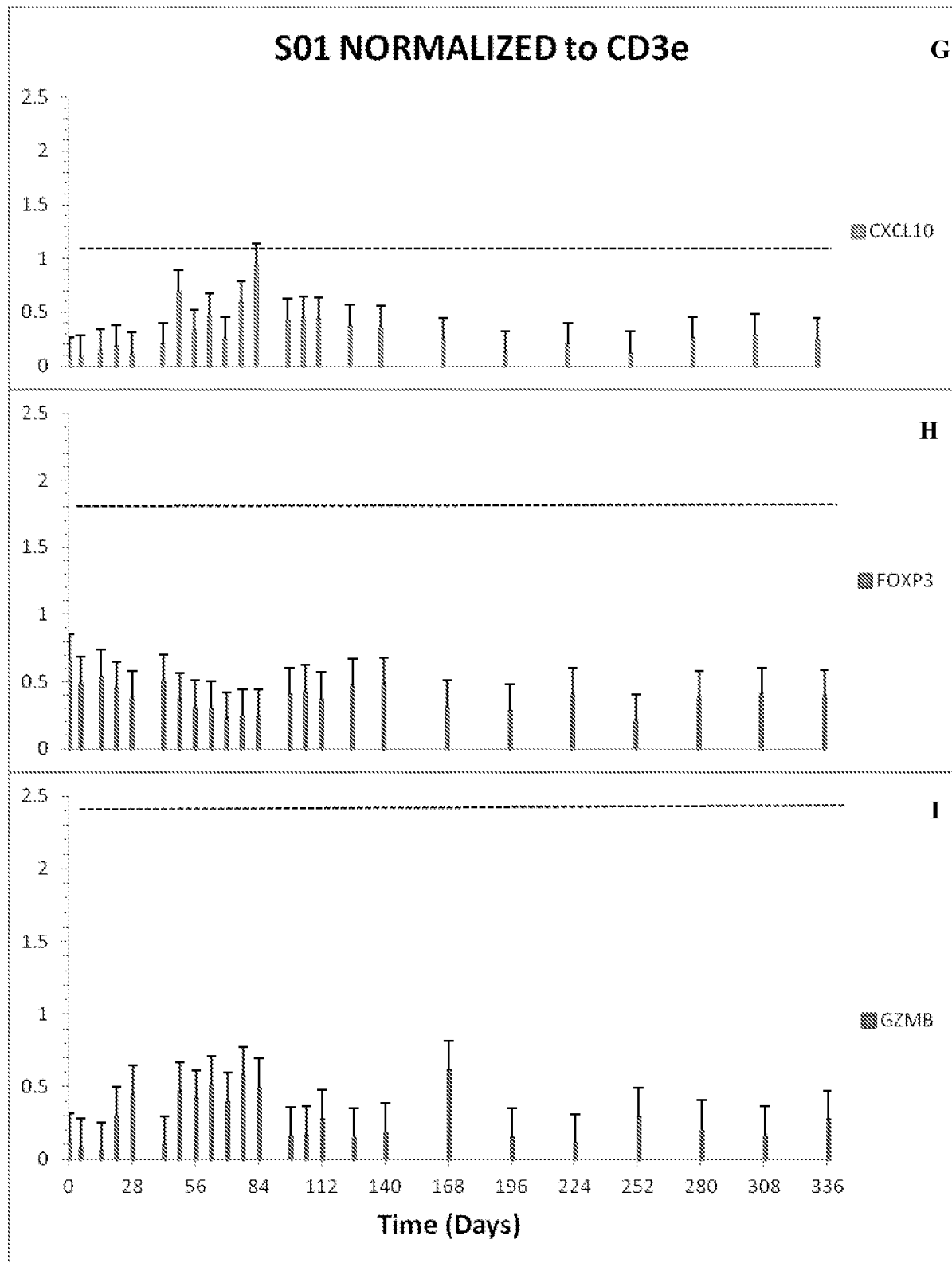


FIG. 3D-F

**FIG. 3G-I**

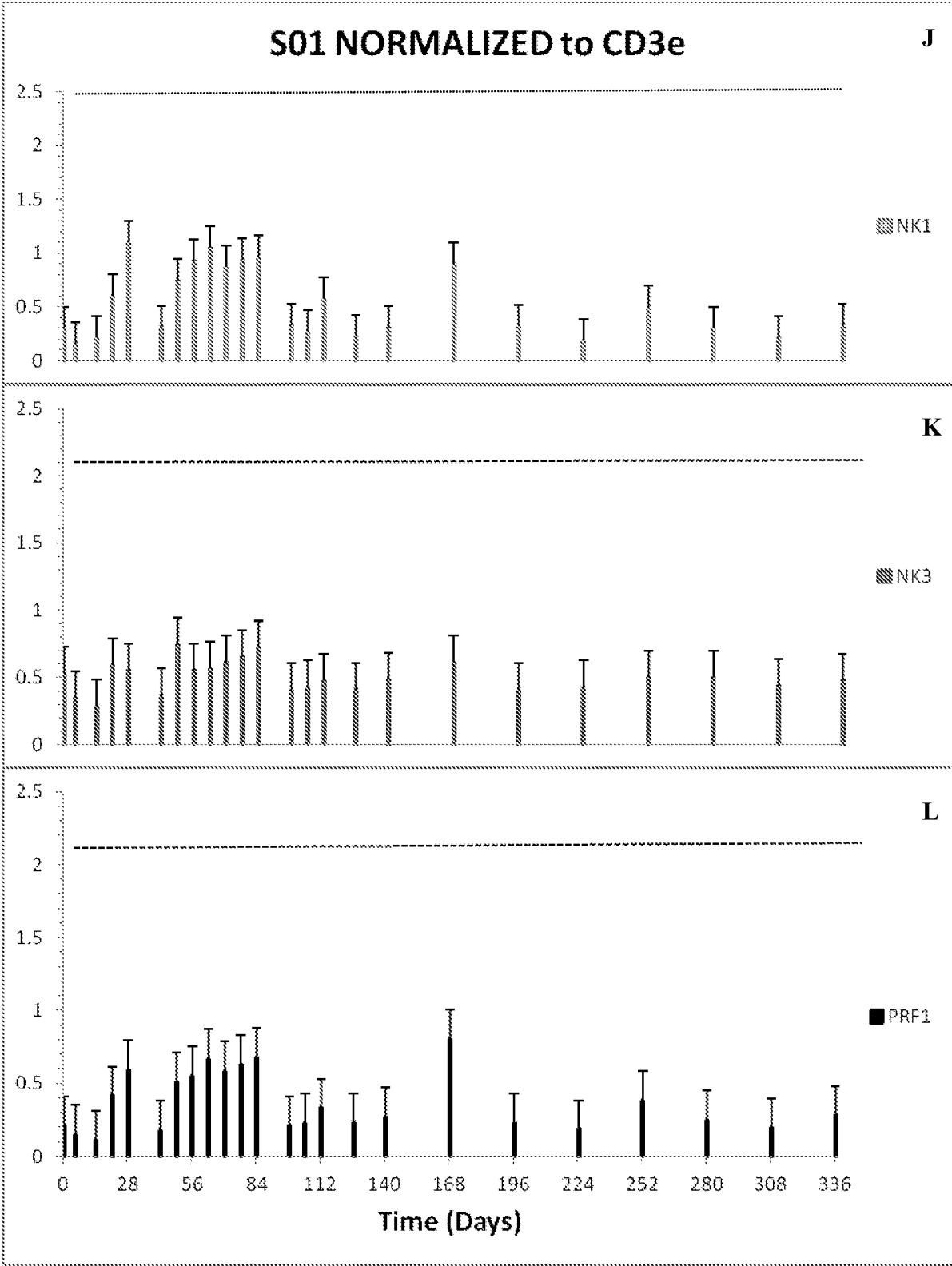
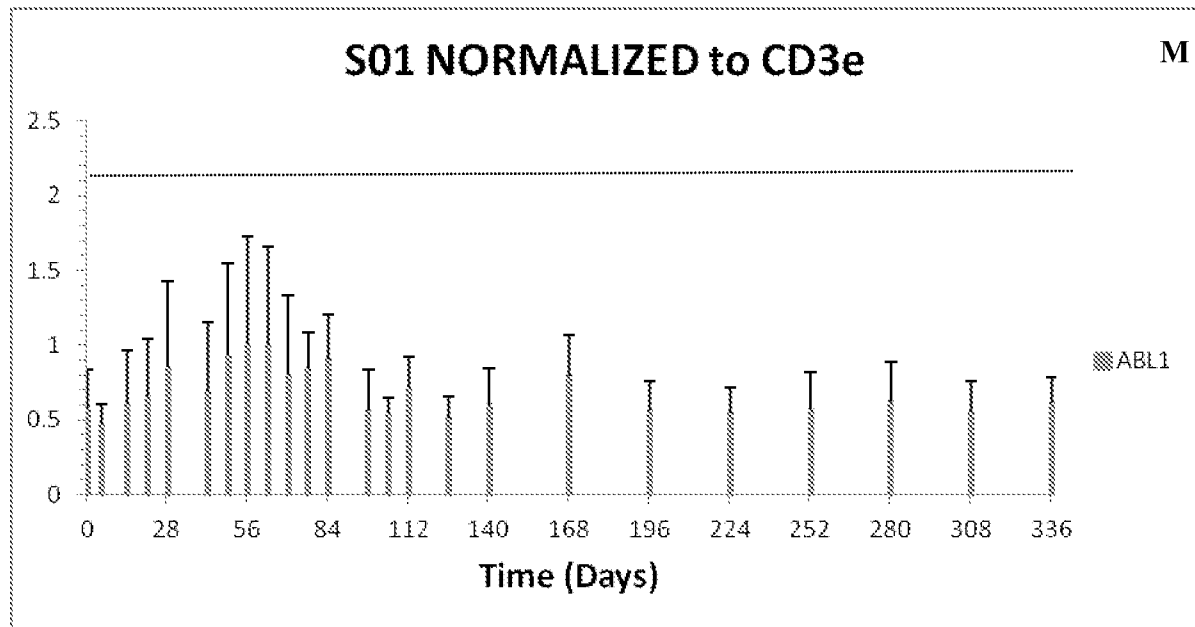
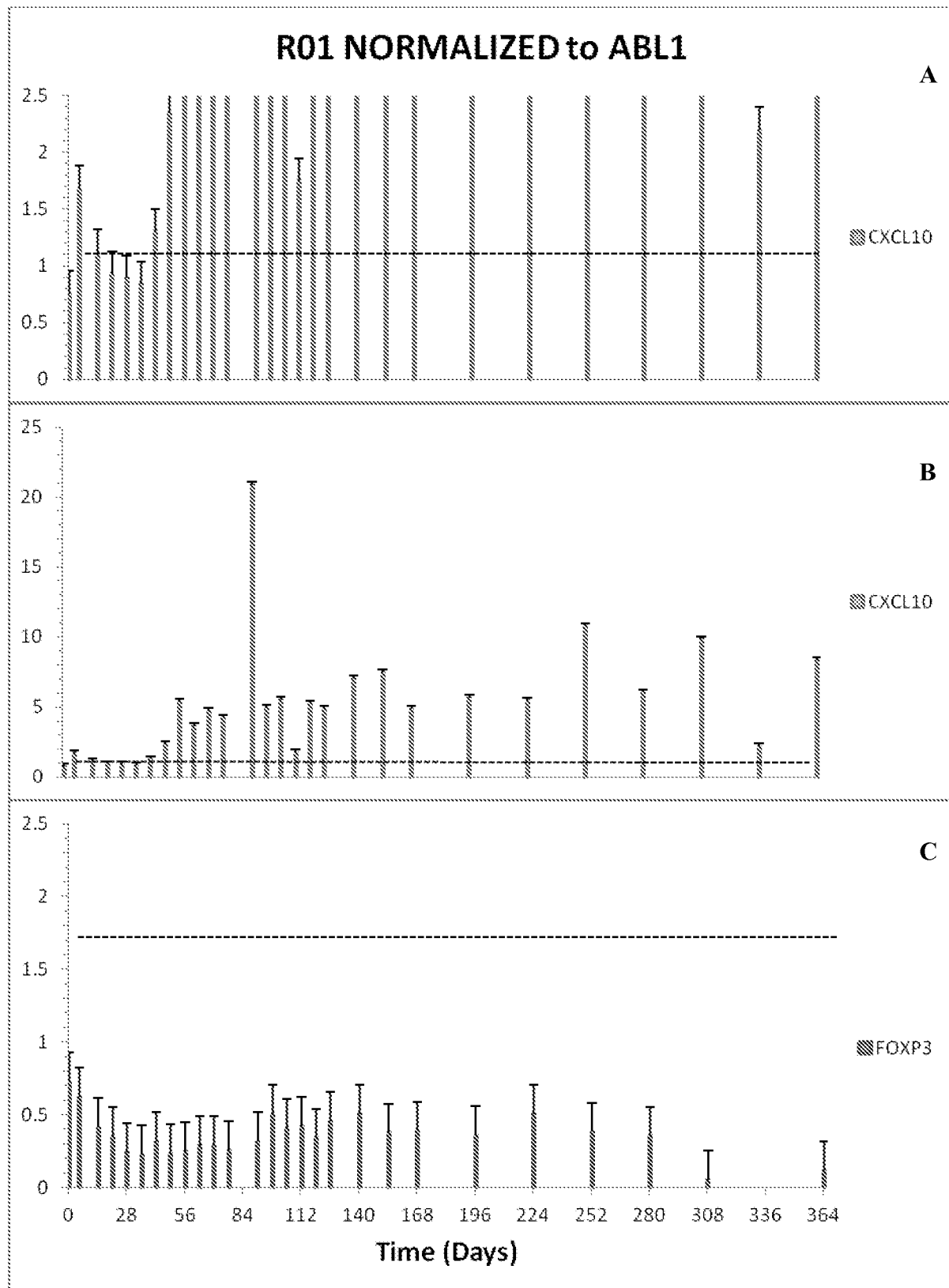


FIG. 3J-L

**FIG. 3M**

**FIG. 4A-C**

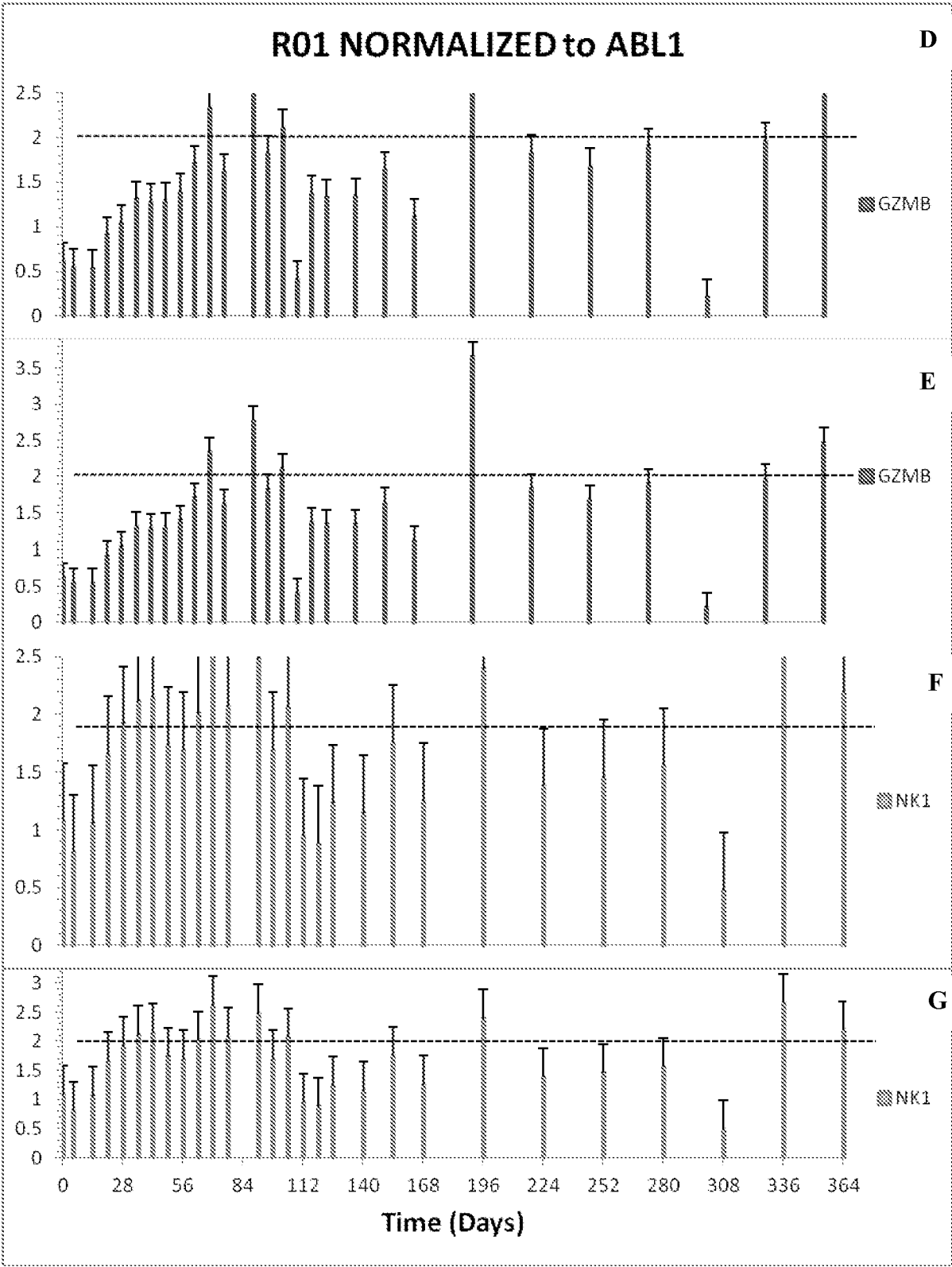
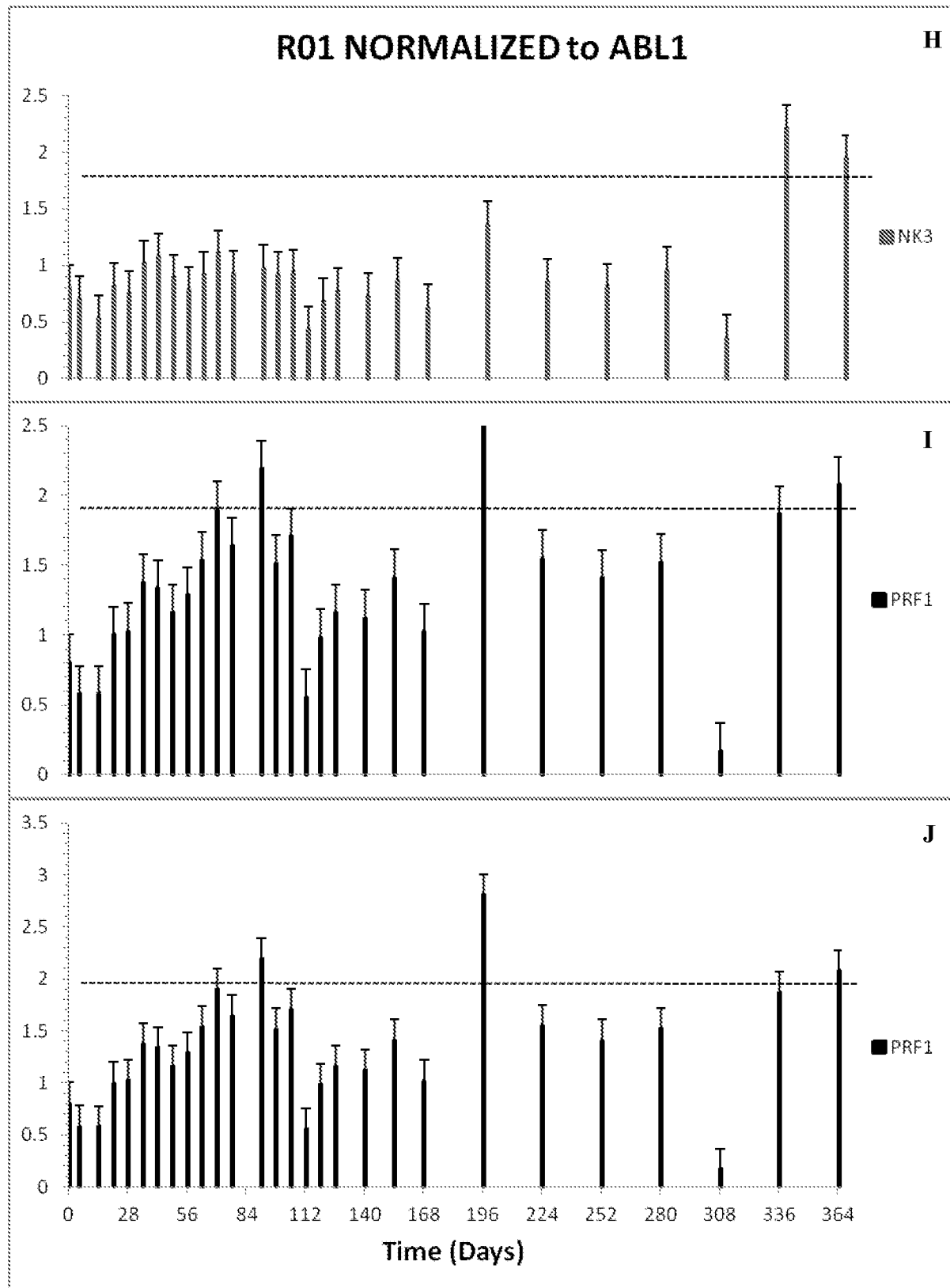


FIG. 4D-G

**FIG. 4H-J**

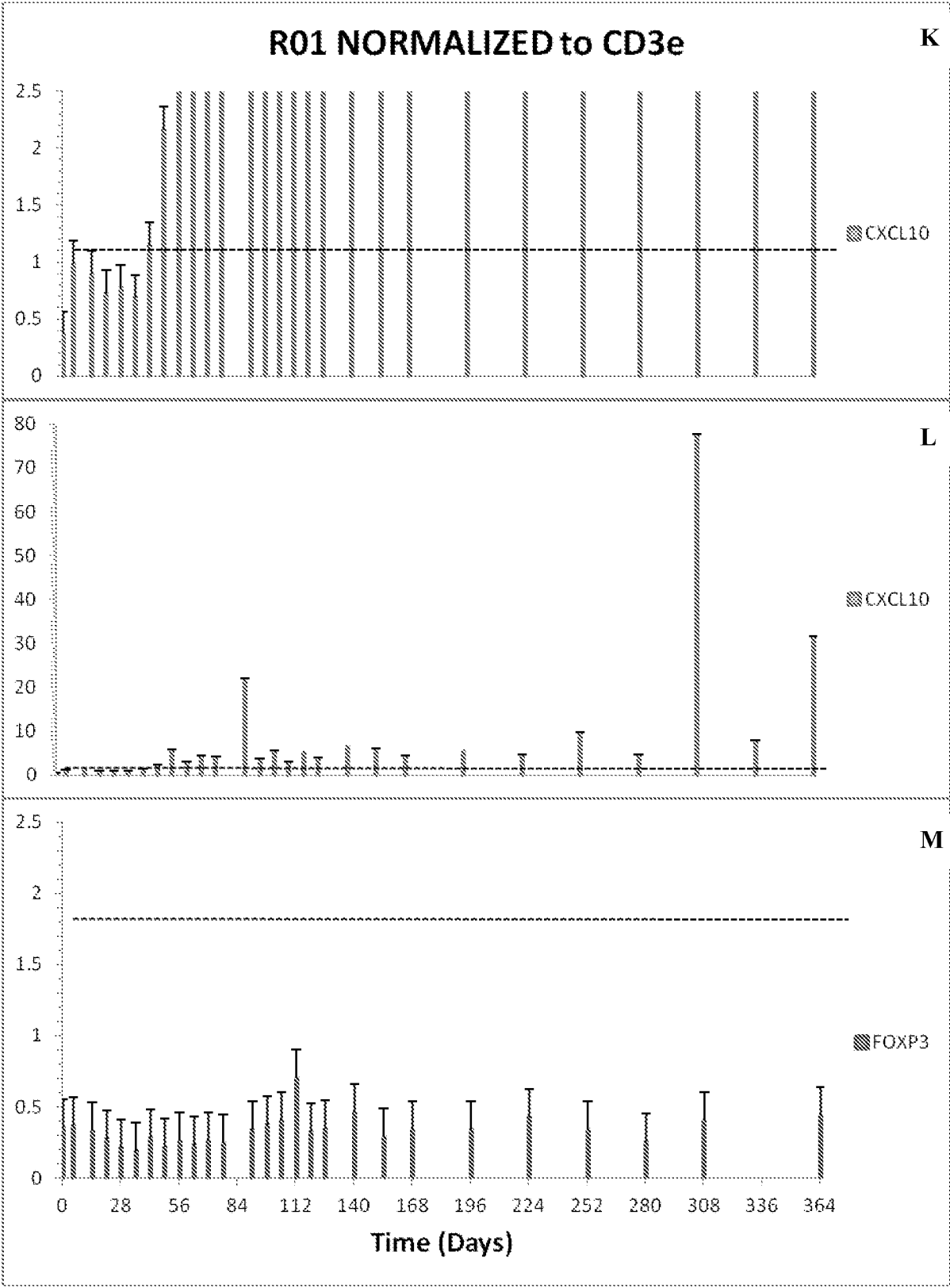


FIG. 4K-M

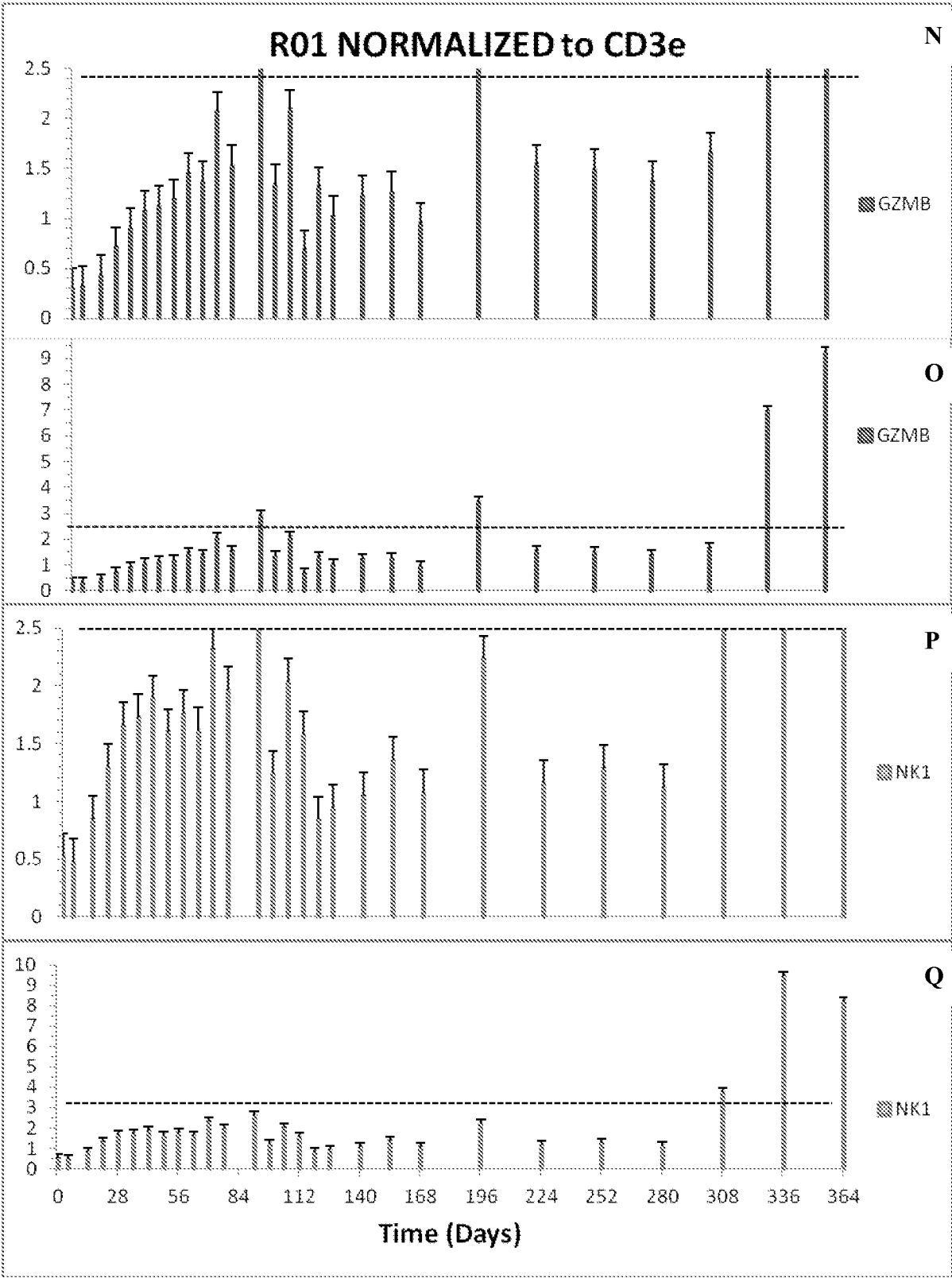
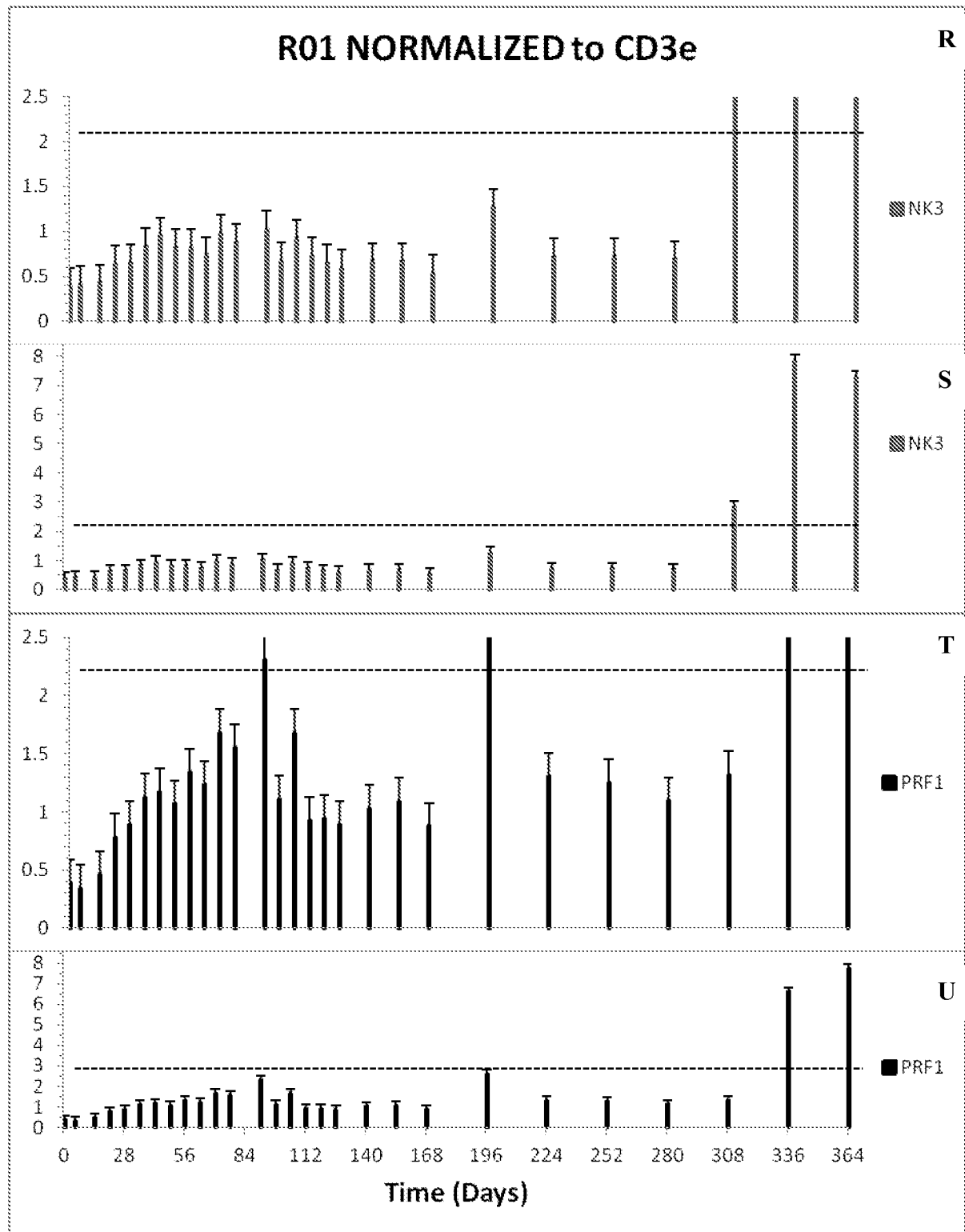
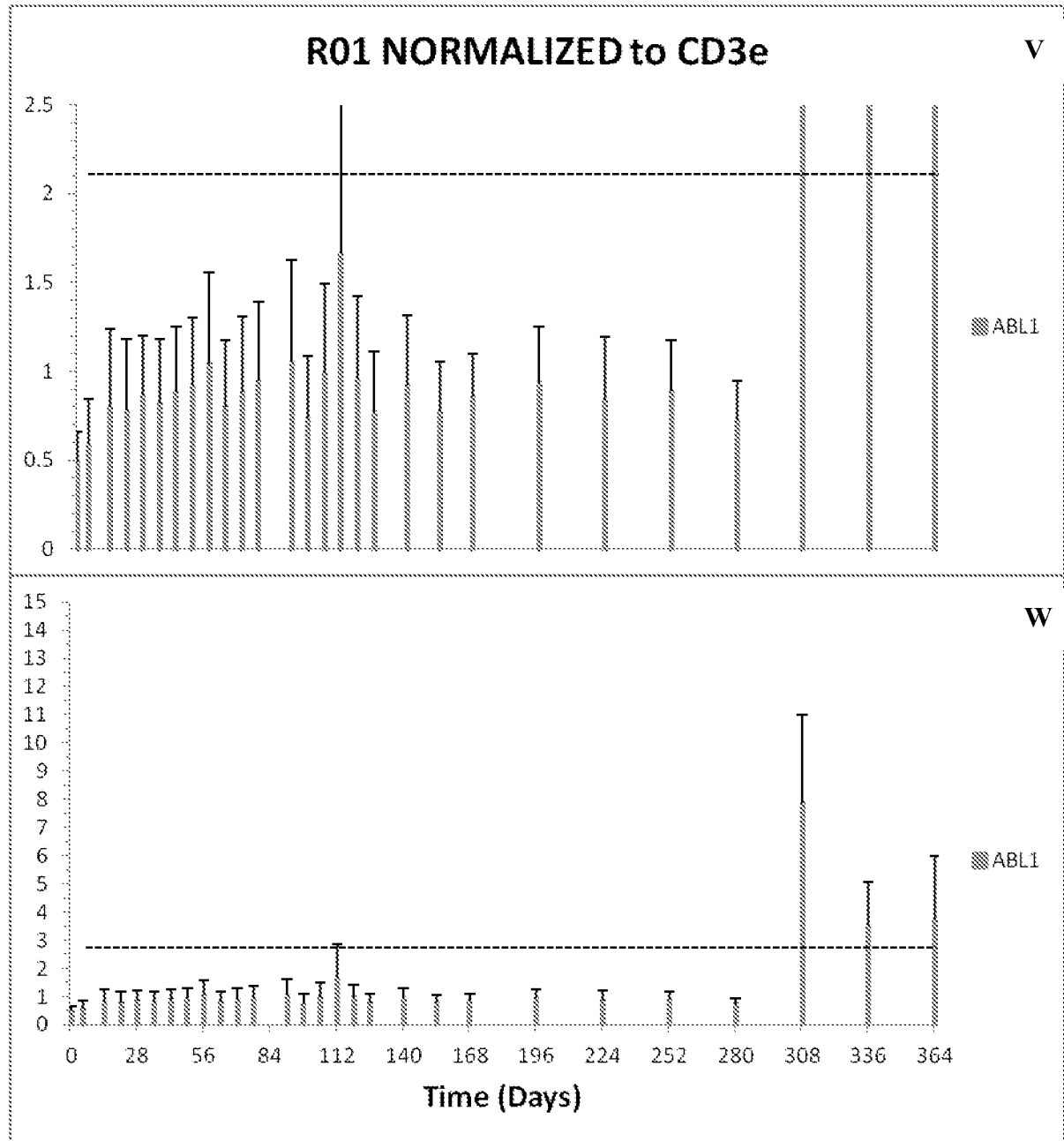


FIG. 4N-Q

**FIG. 4R-U**

**FIG. 4V-W**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US13/68456

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/00, 1/68 (2014.01)

USPC - 435/4, 6.1

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)

IPC(8): C12Q 1/00, 1/68 (2014.01)

USPC: 435/4, 6.1

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

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

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google scholar; Pubmed; ScienceDirect; NCBI GEO; Search Terms Used: Biomarker, ACR, acute cellular rejection, ATN, acute tubular necrosis, renal graft, renal transplant, kidney, creatinin, CRT, granzyme B, perforin, abl1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6187534 B1 (STROM, TB et al.) February 13, 2001; column 1, lines 23-42; column 6, lines 53-55; column 10, lines 18-22	1-4, 6/3, 6/4
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Y		17-20
---		---
A		5/3, 5/4
Y	BROUARD, S et al. Identification Of A Peripheral Blood Transcriptional Biomarker Panel Associated With Operational Renal Allograft Tolerance. PNAS. 25 September 2007, Vol. 104, No. 39; pages 15448-15453; page 15448, left column, first paragraph; page 15450, Table 1. DOI:10.1073/pnas.0705834104.	17-20
A	MEYER-MONARD, S et al. Broad Molecular Screening Of An Unclassifiable Myeloproliferative Disorder Reveals An Unexpected ETV6/ABL1 Fusion Transcript. Leukemia. 24 March 2005, Vol. 19, ppages 1096-1099; page 1098, right column, third paragraph. DOI:10.1038/sj.leu.2403697.	5/3, 5/4
A	ALFIERI, A et al. Role Of NK1 Receptors On Cisplatin-Induced Nephrotoxicity In The Rat. Naunyn-Schmeideberg's Arch. Pharmacol. 18 December 1999, Vol. 361, pages 334-338; abstract; page 443, right column, first paragraph. DOI: 10.10071/s002109900196.	5/3, 5/4

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 January 2014 (21.01.2014)

Date of mailing of the international search report

28 JAN 2014

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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Facsimile No. 571-273-3201

Authorized officer:

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US13/68456

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.