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Development of a Flow Cytometry–Based Functional Assay to Study Anti-TNF Mechanisms of Action and Capture Donor Heterogeneity

Celia Cartagena García, Alexandra Lefèvre, and Jean-Marc R. Busnel
Beckman Coulter Life Sciences, Research Department, Marseille, France

ABSTRACT
TNF is a key cytokine in autoimmune diseases like rheumatoid arthritis, and TNF antagonists are commonly prescribed therapeutics. Although anti-TNF drugs have enabled a very significant progress in this field, disease heterogeneity remains and causes diversity in patient response. These challenges increase the need for anti-TNF characterization tools that may open perspectives toward the development of personalized medicine. In this study, we present a novel whole blood–based flow cytometry functional assay that allows, within a given whole blood sample, the characterization of an anti-TNF molecule mechanisms of action. Whole blood from healthy human donors was employed to mimic the physiological state but also to streamline experimental workflows. Samples were incubated with LPS alone or in combination with various anti-TNF molecules such as adalimumab (ADA), etanercept (ETA), and infliximab. A 10-color flow cytometry panel including CD69, transmembrane TNF, CD16, CD62L, CD66b, CD11b, and CD54 as activation markers was used following a centrifugation-free protocol. CD69 expression decreased on NK, NKT, and T cells upon treatment with ADA, ETA, and IFX as a direct indication of forward signaling neutralization. Percentages of transmembrane TNF+ monocytes increased after incubation when using ADA or IFX but not ETA, revealing the potential of the two first molecules to trigger reverse signaling. Ab-dependent cell cytotoxicity was informed by CD16 and CD69 expressions in some donors that showed increasing levels of CD16+CD69+ NK cells when incubated with anti-TNFs. This study proposes a novel approach to assess anti-TNF mechanisms of action and provides a path toward capturing donor heterogeneity.

INTRODUCTION
Autoimmunity is defined by a loss of tolerance against self-antigens, leading to a dysregulated immune response (1). Women are harshly affected, and prevalence as well as incidence are rising nowadays (2, 3). Onset of these diseases is not fully understood, but either genetic or environmental factors can be determinant to an autoimmune disease promotion. Despite diversity in the field, many autoimmune diseases are characterized by an inflammatory state that leads to the development of chronic diseases, such as rheumatoid arthritis (RA) or Crohn disease (4).

TNF-α is thought to be a major contributor of inflammation in many autoimmune diseases. It is considered a proinflammatory cytokine produced upon infection as a result of cell activation. Monocytes and macrophages are the principal TNF-producing cells, although some lymphocyte subsets and neutrophils are also
capable of producing this molecule. In short, TNF is expressed at the surface of producing cells (transmembrane TNF, tmTNF) and subsequently cleaved by the TNF-converting enzyme, forming the soluble TNF (sTNF), the mature form. Both forms are biologically active and exert their actions through two different TNF receptors, TNFR1 and TNFR2, which are active in soluble and transmembrane forms (5) (6).

TNF acts through two different pathways: forward signaling (FS) and reverse signaling (RS). FS can lead to the NFκB gene transcription by the binding of sTNF with TNFR1 or TNFR2, which is the principal pathway inducing an inflammatory, proliferative, and antiapoptosis response in the TNF-responding cells. In addition, these receptors can also activate the MAPK pathway with similar outcomes. Conversely, TNFR1 has a cytoplasmic death domain that can activate the caspase-8- and caspase-3-dependent apoptosis or anti-inflammatory response by the internalization of the sTNF/TNFR1 complex. Although these pathways are activated depending on the cell state, they can be induced by all TNF forms (5, 6). By contrast, RS occurs in TNF-producing cells after the binding of TNFR2 to tmTNF, with the latter acting as a receptor. When triggered, apoptosis and/or proinflammatory cytokine suppression occur (5, 6). Proinflammatory cytokines are thought to decrease, while anti-inflammatory cytokines, especially TGF-β (7) or IL-10 (8, 9) have been reported to be produced. This mechanism engages TNFRs and IL-10 in autocrine feedback loops, which eventually lead to the downregulation of TNF expression (10).

Because of its pivotal role, the TNF molecule has been the focus of many therapeutic strategies in the 1990s. Since then, it has proven to be a very attractive target to treat autoimmune diseases. The introduction of anti-TNF molecules was indeed a revolution in the field as it represented a very valuable alternative to conventionally used therapies. Nowadays, anti-TNF biologics are some of the top best sellers drugs and are used to treat RA but also other inflammatory conditions such as Crohn disease (11). Blocking TNF receptors mediated FS through binding with sTNF or tmTNF is one of the main mechanisms of action (MOAs). As a function of the molecule, RS and/or Ab-dependent cell cytotoxicity (ADCC) can also be triggered in response to tmTNF binding. Monoclonal IgG1 Abs like adalimumab (ADA), infliximab (IFX), and golimumab but also the IgG1–Fc fusion protein etanercept (ETA) and the humanized Fab′ PEGylated certolizumab pegol are popular TNF blockers. Because of the enormous market behind anti-TNFs, many biosimilars such as Hulio protein etanercept (ETA) and the humanized Fab′ PEGylated certolizumab pegol are popular TNF blockers. Because of the enormous market behind anti-TNFs, many biosimilars such as Hulio

### MATERIALS AND METHODS

#### Samples

In the current study, blood from volunteers visiting St. Joseph Hospital (Marseille, France) was used with the previous consent of the donors. Blood was collected in heparin-coated (spray dried) Vacutainer tubes (BD Biosciences) to avoid metal ion sequestration.

#### Flow cytometry–based functional assay

To study extracellular marker expression, whole blood samples were initially activated with LPS (Sigma-Aldrich) at a final concentration of 200 ng/ml. Fifty microliters of freshly diluted ADA (AbbVie), ETA (Amgen), or IFX (Merck) in a water bath for 4 h at 37°C. For each sample, a negative control and an LPS control were included in addition to the conditions with anti-TNFs. The volume was kept constant throughout the conditions by adding PBS. To confirm the capability of our approach to assess ADCC, obinutuzumab (Roche), a well-known afucosylated anti-CD20 with very strong NK cell engaging abilities was also employed as a positive control (Supplemental Fig. 1). Cell viability was also assessed in this study to ensure that our incubation conditions did not significantly alter this parameter (data not shown).

After incubation, cells were stained for 30 min in the dark at room temperature with the following staining mix: CD3-PE (Becton Dickinson), CD4-PerCP-Cy5.5 (BD Biosciences), CD8-APC-H7 (BD Biosciences), CD14-PE-Cy7 (BD Biosciences), CD45-PE-Cy7 (BD Biosciences), CD16-AF488 (BioLegend), and CD56-AF647 (BioLegend). Cells were then washed and fixed for flow cytometry analysis. To assess ADCC, blood samples were incubated with ABC-PE (Beckman Coulter Life Sciences) and then stained with CD16-PE-Cy7 (BD Biosciences) and CD56-PE-Cy7 (BD Biosciences).
manufacturer in the OptiLyse C instructions for use, 250 µl of OptiLyse C were added to the incubated whole blood samples followed by an immediate and vigorous vortexing step before letting it react for 10 min. Two hundred and fifty microliters of PBS were then added to the mix. After 10 min and within 3 h, surface marker expression was measured with a three-laser 13-color CytoFLEX flow cytometer (Beckman Coulter Life Sciences). Data were analyzed according to the gating strategy displayed in Fig. 1.

**Statistical analysis**

Analysis of flow cytometry data was performed with Kaluza Analysis 2.1. (Beckman Coulter), GraphPad Prism 8.3 (GraphPad software) and JMP 14.2 (SAS Institute) were used for statistical analysis, marker correlation, and clustering. Percentages of populations or arithmetic mean fluorescent intensity (MFI) were considered (mean percentages or MFI). Differences in percentages or MFI were evaluated by a nonparametric t test, and statistical significance was established with the following p values: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

**RESULTS**

The panel used in this study was carefully designed, aiming to capture all anti-TNFs MOAs. Despite a huge variety of potentially useful activation markers, in this study, it was decided to focus on a single and relatively simple panel to attempt demonstrating the proof of concept. Besides lineage markers (CD3, CD14, CD16, CD45, and CD56) that were used to discriminate the major immune cell subsets (as displayed in Fig. 1), CD69 and CD11b were used as early activation markers and thought to be useful to assess FS on a variety of leukocytes subsets (15, 16), whereas CD66b (CEACAM8) was included to more specifically investigate neutrophil activation. TNF was included so that the increase of tmTNF could be assessed as an indicator of anti-TNF binding and possible RS triggering. Along with CD11b, CD54 (8, 17) and CD62L (18, 19), also known as ICAM-1 and L-selectin, respectively, are other adhesion molecules that have already been demonstrated to be of interest in pathologic conditions such as RA.

![FIGURE 1. Gating strategy based on CD45, CD14, CD3, and CD56 marker expressions.](https://doi.org/10.4049/immunohorizons.2000077)

CD45+ cells were first gated to consider only leukocytes. Lymphocytes, monocytes, and granulocytes were identified by the side scatter and the expression of CD14. NKC, NKCD56+, NKCD56dim, NKT, T, and CD3–CD56– cells were selected from the lymphocytes according to CD56 and CD3 expressions.

![FIGURE 2. CD69/CD11b expression in several cell subsets upon LPS-based activation of whole blood.](https://doi.org/10.4049/immunohorizons.2000077)

Case of a representative donor. FACS dot plots depict nonstimulated and LPS-stimulated samples. The expression of CD69 and CD11b is represented for NK, NKT, T, monocytes, CD3–CD56– cells, and granulocytes.
Activation profiles of the major cell subsets upon whole blood stimulation with LPS

LPS is a well-known in vitro monocyte activator through TLR4-specific binding. It is also known that B cells can be directly activated through this pathway. However, Fig. 2 shows, for a representative donor, how LPS stimulation in whole blood not only induced monocyte and B cell priming but also the activation of the vast majority of leukocytes, as previously reported for some lymphocyte subsets (20, 21). This systemic activation is certainly a consequence of a signaling cascade initiated at the monocyte level, which, upon LPS activation, may produce massive amounts of cytokines, TNF included.

As observed in Fig. 2 in the case of a specific donor, the magnitude of the LPS effect varied depending on the cell type. When considering CD69 and CD11b, it appears that NK cells showed the highest activation, shifting from ~11% CD69+CD11b+ cells in the non-stimulated sample to 91% with LPS. NKT cells were also strongly activated as illustrated by the CD69+CD11b+ cell proportion that augmented to ~49% in the LPS condition. However significant, the magnitude of the activation tended to be lower in other subsets such as T cells, monocytes, granulocytes, or CD3-CD56- cells with the latter certainly mostly constituted by B cells.

Anti-TNF MOAs can be assessed by CD69, CD11b, tmTNF, and CD16 expression

To determine whether anti-TNF MOAs can be assessed with the extracellular panel designed, whole blood was activated with LPS and further incubated for 4 h at 37°C in the absence or presence of ADA, ETA, or IFX. A negative control sample was also incubated at 37°C for 4 h without LPS nor anti-TNF biologics.

After studying the different LPS activation profiles in Fig. 2 and the possibility for other cell subsets than monocytes to be stimulated under such conditions, Fig. 3 illustrates, in the case of representative donors, how MOA triggering by the different anti-TNF biologics could be assessed. NK cells were selected to study FS further as they were among the most intensively activated cells. As described above, increasing percentages of CD69+NK cells from NS to LPS-stimulated samples were found together with a brighter expression of CD11b. When treating samples with ADA, ETA, or IFX, percentages of positive

![Image: Anti-TNF MOAs identified by CD69, CD11b, CD16, CD54, and tmTNF expression.](https://doi.org/10.4049/immunohorizons.2000077)
CD69 and CD11b<sup>bright</sup> cells decreased significantly. It is believed to be a consequence of FS neutralization. Although this effect was observed for the three biologics tested, it was the strongest in the case of IFX. In parallel to FS inhibition, we also attempted to study the triggering of RS by considering both tmTNF and CD54 expressions on monocytes. An increase of tmTNF<sup>+</sup> cells was observed in ADA- and IFX-treated samples. Conversely, no increase was found in the ETA condition. This observation is in agreement with the fact that ADA and IFX are well-known RS triggers, whereas ETA is not (5). Of note, CD54 expression had the exact opposite behavior, observing a decrease after ADA and IFX but not after ETA treatment. Apart from mediating RS, tmTNF accumulation in the TNF-producing cells might also enable ADCC when TNF blockers bind to it. ADCC is a mechanism by which effector cells such as NK cells are engaged toward the lyse of anti-TNF-bearing target cells. This mechanism engages the Fc portion of the anti-TNF molecules that bind with high affinity to CD16 on NK cells. Hence, the decrease of CD16 and simultaneous increase of CD69 expressions on NK cells could be used to assess this mechanism as showed in Supplemental Fig. 1 in our obinutuzumab-positive control. A representative donor that did show a decrease in CD16 expression upon ADA and ETA treatment is represented on Fig. 3. The characteristic shape of ADCC triggering was seen in ADA and ETA but less prominently in the case of IFX. NK<sup>CD56<sup>dim</sup></sup> cells were selected to study this mechanism as CD56-bright NK cells do not contain CD16 on their membrane (22).

**Anti-TNF dose dependency and repeatability of observed processes**

To assess the repeatability of our assay and better assess the dose dependency of monitored MOAs, experimental triplicates were performed using IFX at different doses in a dose-response fashion.

For all considered parameters, most of the evolution occurred with doses below 1 µg of IFX with parameters reaching a plateau in most of the cases beyond this point as depicted in Fig. 4. Of note, MFIs did show an elevated coherence between triplicates with relative SDs (RSDs) ranging from 0.4 to 16.5%, with the highest variability observed at 3 and 10 µg for all parameters. At the working concentration of 1 µg, RSDs between 0.9 and 2.7% were observed for CD69, CD11b, and CD16, whereas RSDs ranging from 12.8 to 13.5% were obtained for CD54 and tmTNF.

**FS neutralization upon ADA, ETA, and IFX treatment and heterogeneity of response between donors**

The approach described beforehand was further implemented in 20 different whole blood samples in an attempt to evaluate the donor heterogeneity of response.

Focusing on FS inactivation, frequencies of CD69<sup>+</sup>CD11b<sup>+</sup> cells were initially studied in NK, NKT, and T cells (Fig. 5). Although a significant effect was observed on these different populations, NK cells revealed the most prominent differences. Subsequently to an increase of CD69<sup>+</sup>CD11b<sup>+</sup> cells upon LPS activation, percentages decreased significantly in all donors when ADA, ETA, and IFX were added into the incubation mix. NKT cells and T cells exhibited a similar effect but of a lower magnitude. By contrast, CD<sup>3</sup>CD<sup>56</sup><sup>+</sup> cells, monocytes, and granulocytes did not present any significant statistical differences in these parameters. Still, taken individually, a noticeable difference in donor responses could be observed, especially in monocytes. For granulocytes, CD66b was additionally investigated. Although a slight decrease upon ADA treatment could be detected, no statistically significant differences were found after any anti-TNF treatment in comparison with the LPS condition (Supplemental Fig. 2).
**RS triggering and donor heterogeneity of response**

After investigating TNF-responding cells, TNF-producing cells were also studied in more detail. More specifically, monocytes, as one of the greater producers of TNF, were gated according to SSC and CD14 expression (as showed in Fig. 1).

It is known that the TNF-converting enzyme cleaves tmTNF from the cell surface and converts it into sTNF, the mature form. This is in accordance with the observation that no tmTNF is detected on monocytes despite an LPS activation (Fig. 6). Conversely, tmTNF was detected at the surface of monocytes when samples were incubated with ADA or IFX. No increase of tmTNF+ monocyte percentages were found in ETA-treated samples, agreeing with the incapacity of this molecule to trigger RS (Fig. 6). Significant differences between the negative condition and the LPS-stimulated samples were also found; however, detected levels of tmTNF remained very low in comparison with what was observed with ADA or IFX. A possible explanation to this background noise might be related to the temporary presence of tmTNF at the surface of monocytes.

Regarding CD54 expression, a significant increase was noticeable when cells were activated with LPS. Upon the addition of anti-TNF biologics, no significant differences could be detected when considering the group of treated samples as a whole. Nevertheless, a significant heterogeneity could be detected when considering individually the different samples. The average decrease of CD54 expression upon ADA and IFX treatment was 13 and 14%, respectively. However, a few donors showed a decrease of more than 30% with as much as 82 and 76% variation with ADA and IFX in the most extreme case.

**NK cell engagement through ADCC**

Deepening more into the different anti-TNF MOAs, NKdim cells were closely looked into for assessing a possible ADCC induction. Proportions of CD69+CD16- were more specifically studied (Fig. 7).

As compared with the other MOAs, ADCC was more difficult to appreciate. The only biologic that yielded a significant difference with LPS alone was IFX, suggesting that this TNF antagonist is the most susceptible to trigger ADCC. Nevertheless, ADCC was performed by ADA and ETA in two of the samples screened. In parallel, a control with obinutuzumab, an afucosylated anti-CD20 very potent at engaging NK cells through ADCC, was performed to confirm the suitability of this assay to evaluate this MOA (Supplemental Fig. 1).

**FIGURE 5. CD69+CD11b+ cell percentages in NK various cell subsets.**

Nonstimulated, LPS-stimulated, and LPS-stimulated ADA, ETA, or IFX conditions are displayed (n = 20) for NK, NKT, T cells, monocytes, CD3-CD56- cells, and granulocytes. Statistical significance was calculated by a nonpaired, nonparametric Kruskal–Wallis test obtaining levels of significance according to *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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WHOLE BLOOD–BASED FUNCTIONAL ASSAY TO CHARACTERIZE ANTI-TNFs
Correlation between CD11b, CD16, CD54, CD62L, CD66b, and tmTNF expressions

A total of six different activation markers were analyzed in this study. To evaluate how complementary and/or orthogonal they are, a multivariate analysis was performed. All markers (excluding lineage and gating markers) were investigated on NK, NKT, T cells, monocytes, and granulocytes, and correlative features were first explored (Fig. 8). To this end, data obtained on the same cohort of whole blood samples ($n = 20$) as the one considered for Figs. 5–7 was considered.

As observed on Fig. 8, strong correlations were obtained between CD69 on various subsets, especially between NK and NKT cells ($r = 0.7487$) or T cells ($r = 0.7792$) as between NKT and T cells ($r = 0.7227$). CD11b on NK cells also correlated with CD69 on NK ($r = 0.6285$), NKT ($r = 0.5348$), and T cells ($r = 0.4657$). On granulocytes, CD11b was intensely correlated with CD66b ($r = 0.8237$). With a fewer intensity (lighter red color), CD69 and CD54 on monocytes correlated with CD69 on NK ($r = 0.6285$), NKT ($r = 0.5348$), and T cells ($r = 0.4657$). On granulocytes, CD11b was intensely correlated with CD66b ($r = 0.8237$). With a fewer intensity (lighter red color), CD69 and CD54 on monocytes correlated with CD69 on NK, NKT, and T cells. Contrarily, the previous markers negatively correlated to CD62L in monocytes, which grouped in the left bottom of the matrix, forming a different group (medium and deep blue). None or less correlations could be observed between the other analyzed parameters.

Sample clustering depends on activation conditions

Trying to assess whether the proposed approach could represent a path toward patient or donor stratification through exhibiting various behaviors upon stimulation conditions, a hierarchical cluster analysis was then performed. Although the detailed analysis is visible in the supplementary information file (Supplemental Fig. 3), Fig. 9 shows the constellation plots that could be generated from this analysis.

Interestingly, it was found that the clustering was significantly different as a function of the activation condition, thereby demonstrating that interrogating the function of the cells and not only their phenotypes could bring very valuable information. As an example, donors 5 and 12 can be studied more precisely. Although they clustered together in the nonstimulated condition (Fig. 9A), they did not after incubation with LPS, LPS+ADA, LPS+ETA, or LPS+IFX (Fig. 9B–E). Similar examples could be found subsequently, considering other pairs of whole blood samples such as 1 and 8, 1 and 4, or 2 and 3.
**DISCUSSION**

The introduction of anti-TNFs to the market as treatment for autoimmune diseases has changed the therapeutic landscape of the field. Anti-TNF biologics have been proven to significantly reduce disease progression in some diseases, for instance in RA, slowing joint destruction (23) and suitably functioning in monotherapy or in combination with other disease-modifying drugs.

Despite the benefits provided by the wide range of anti-TNFs developed during the past 30 y, heterogeneity between patient features still represents a significant challenge today with satisfactory and long-lasting response to therapy not always being achieved. This fact hinders the design of an adequate therapy for each patient, making the therapeutic approach time consuming and not fully efficient.

With TNF being such a relevant therapeutic target, there is a need for a better characterization of anti-TNF molecules and associated MOAs with a focus on the heterogeneity of response between individuals. In this study, we propose a novel whole blood–based functional assay with the aim of better grasping the latter parameter through providing a model potentially closer to a physiological state. Indeed, we explored the use of peripheral whole blood, in contrast to commonly used PBMC or cell lines. This material not only contains all subclasses of circulating leukocytes but also all other soluble biological materials such as circulating cytokines, Abs, metabolites, and proresolving lipids, all of which have been proven to be meaningful for the study of RA for example. Our objective is also to highlight the use of whole blood as an easy-to-process sample type to carry out in vitro preclinical and/or clinical studies. We believe that such an assay could help better characterize anti-TNF MOAs within a sample’s referential and therefore represent a potential path to further improve current therapeutic strategies.

Functions of therapeutic mAbs have been classically studied by histology, equilibrium dissociation constants (by fluorescence ELISA or kinetic exclusion assays), ligand binding, or cell-based assays, among others (24). In this study, we used flow cytometry as the main technique to investigate anti-TNF biologics MOAs through the monitoring of several activation markers in whole blood. This strategy relies on an initial TLR4-mediated stimulation with LPS upon which monocytes are specifically activated. Although it is a well-known and broadly used activation approach for in vitro cell stimulation studies, we found it of particular interest for the study of anti-TNF therapeutic MOAs as it not only triggers a massive production of TNF by monocytes but also yields a highly inflammatory environment. Indeed, taking advantage from whole blood, other cell types subsequently respond, downstream of monocyte activation, as indicated by our results and as previously described in the literature with PBMCs (20, 21). This activation cascade is made possible by the ability of monocytes to produce TNF and other proinflammatory cytokines, such as IL-6, IL-8, or IL-1ß, etc., thereby resulting in an inflammatory environment in which a significant proportion of cells are activated. Our working hypothesis is that such a scenario could hypothetically translate into what is happening in vivo during pathological inflammatory responses, thereby providing foundations to study in vitro the ability of an anti-TNF biologic to trigger various MOAs within an individual’s referential.

Not only monocytes but most cell subsets become activated upon LPS stimulation. In our case (Fig. 2), NK cells underwent the strongest increase of CD69. However, NKT cells, T cells, CD3− CD56− cells, and granulocytes also exhibited increased CD69 expressions. TNF together with other proinflammatory cytokines and/or mediators may play a role in the activation cascade initially triggered by monocytes and their reactivity to LPS. If mediated by TNF, the expression of both TNFRs on each of these subsets may influence the magnitude of activation (25, 26).

Following our initial results, we then embarked on studying ex vivo the different MOAs of anti-TNFs: FS inhibition, RS triggering, and ADCC. Data from this study prove that all three MOAs can be assessed using the developed approach. In brief, very broadly expressed activation markers such as CD69 and CD11b, when monitored on NK, NKT, or T cells, inform about FS inhibition. The level of tnTNF on monocytes informs about the ability of the tested biologics to trigger RS. The expression of both CD69 and CD16, when concomitantly studied on CD56dim NK cells, provides information about the engagement of NK cells through ADCC.

Even though a significant heterogeneity of response was observed, FS inhibition and RS triggering could be illustrated in all donors tested. In contrast, it was only possible to capture ADCC in a few of them. ADCC triggering actually depends on several factors, including the proportion of TNF-producing cells, the

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**FIGURE 8. Heatmap based on correlation coefficients between MFIs (n = 20).**

Correlations are based on the row-wise method, in which missing data on any variation is excluded from the calculation. Maximum correlation coefficient is represented in deep red (r = 1), and minimum correlation in deep blue (r = −1).

![Heatmap](https://doi.org/10.4049/immunohorizons.2000077)
density of tmTNF at their surface, and the CD16 phenotype of the considered donor. It is also well known that the Fc region of the considered mAb further plays a central role in regulating the affinity of the Ab for the Fc receptors (27). To ensure that the relative inability to detect ADCC under our conditions was not due to the analytical approach implemented but rather to parameters relative to the tmTNF target and/or to the donors tested, obinutuzumab was also considered. By including this afucosylated IgG1 anti-CD20 mAb, which is known to be a very potent ADCC engager, we could confirm the assay’s capacity to capture this MOA.

Studying in further detail the activation phenotypes, depending on the incubation conditions, revealed some other interesting features and enabled gathering additional insights. As observed in Fig. 5, percentages of CD69+ granulocytes, which are not known to be directly activated by LPS, were maintained following incubation with TNF blockers. This behavior could potentially be explained by the fact that another molecule/cytokine than TNF, such as IL-8 (28), might be responsible for the activation of granulocytes under the experimental conditions tested. We also observed that CD69 did not correlate with CD66b in any significant extent ($R = 0.12$). Interestingly, CD66b expression showed differences when ADA and IFX were present during the incubation, suggesting a link with the RS MOA. Among the other markers, CD66b correlated the most with CD11b on granulocytes ($R = 0.82$) and CD54 on monocytes ($R = 0.48$). The latter is expressed constitutively on monocytes and has been shown to be upregulated during inflammatory conditions (29). This is coherent with the ability of RS to alternatively provoke apoptosis or decrease of proinflammatory cytokines production, including IL-8. RS can change the proinflammatory phenotype of monocytes toward an anti-inflammatory profile through IL-10 production and/or proinflammatory cytokine production mitigation. The fact that such behavior was not systematically detected in all donors tested further illustrates a significant donor heterogeneity in triggering the RS MOA.

Although CD69, CD16, and tmTNF were included to inform about the MOAs of TNF blockers, CD11b, CD54, CD62L, and CD66b were also considered. Those are adhesion molecules and have critical roles in leukocyte migration and infiltration during in vivo inflammatory conditions (30–33). Additionally, CD54 and

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**FIGURE 9.** Sample clustering pattern of the 20 donors included in the study. Negative control without stimulation (A), LPS condition (B), and the three anti-TNFs, (C) ADA, (D) ETA, and (E) IFX, conditions normalized by LPS are depicted. Clusters are represented by colors in an arbitrary scaling. Each row is displayed by an end point, and each cluster join is represented by a new point. Lines represent distance between clusters and are relative to each other.
CD62L have been shown to be potentially relevant in RA. Whereas the expression of CD54 on monocytes decreases in patients with a good therapeutic response to ETA (8), CD62L expression decreased on NK cells from RA patients (18), although its expression on CD45RA -CD8 T cell central memory population was increased (19) in these same category of patients. To streamline the assay, one can interrogate whether all of the markers considered for this study would be required in a follow-up work. A multivariate analysis was thus performed, and no significant redundancy between the markers was found. Hence, we believe that the study of these markers could be informative, if not decisive, in future studies aiming at leveraging the proposed assay.

In summary, our functional assay model based on flow cytometry proposes a novel approach to inform about the capabilities of a donor to respond through various anti-TNF MOAs. One of the intents of initially working with whole blood samples from healthy individuals not receiving anti-TNF therapies was to assess our capabilities to induce ex vivo an inflammatory situation that would enable the study of anti-TNF MOAs on easily accessible samples. Considering that pathological sample procurement may sometimes represents a challenge, we indeed believe that such a possibility could facilitate the realization of important preclinical studies. Further research has now to be performed to evaluate whether biopharmaceutical characterization and/or personalized medicine for autoimmune disease therapy could benefit from this approach. Nowadays, translational researcher’s efforts indeed focus on improving the patient’s quality of life with personalized therapies. However, analytical strategies to assess and predict patient response to a drug are lacking. Following our research, we found different clustering patterns depending on the conditions of stimulation, which demonstrates that individuals cannot only be stratified as a function of their baseline immune system but also as a function of their ability to respond ex vivo to various therapeutics. For these reasons, we do believe that such functional approaches represent valuable paths to explore further in the context of both biopharmaceutical characterization and clinical research. With this proof-of-concept study, we aimed to develop and further demonstrate the capabilities of our whole blood–based functional assay to study anti-TNF MOAs. This is the first step only; further studies including RA patient’ cohorts receiving or not anti-TNF therapies now need to be conducted as well as extensive clinical validation to probe and further characterize the possible clinical relevance of this assay.

DISCLOSURES

All authors are past or current employees of Beckman Coulter Life Sciences.

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