Clinic Rev Allerg Immunol (2017) 53:177–180
DOI 10.1007/s12016-017-8604-9

CrossMark

Immunophenotyping As a New Tool for Classification and Monitoring of Systemic Autoimmune Diseases

Yves Renaudineau [1,2]

Published online: 5 May 2017
© Springer Science+Business Media New York 2017

Abstract The clinical course of systemic autoimmune diseases (SADs) varies greatly, even between individuals with the same disease. Understanding of the immune actors is informative and could lead to significant improvements in diagnosis, monitoring, initial treatment decisions and/or follow-up. However, immunological changes in mononuclear cells associated with SADs have been only partially described, and usually are limited to analysis of peripheral blood cells (less than 5% of the total mononuclear pool). Another limitation is technological, related to utilization of flow cytometry, which remains highly variable with regards to sample preparation, reagents, instrument constraints, and data analysis. As a consequence, and although confirmation conducted by independent teams using multivariate analysis is lacking for proposing to use immunophenotyping in the diagnosis and/or follow-up of patients, there is a consensus of interest for monitoring several mononuclear cell subsets for emerging roles in SADs including memory B cells, effector T cells, and dendritic cells. In the near future and with the development of next generation technologies and standardized operating procedures, it is predicted that flow cytometry will find its place in the development of future personalized medicine in SADs. In addition, better understanding of immunological deregulations (e.g., intracellular phosphoproteins and cytokines, calcium actors) in both human and SAD-prone mouse models, as presented in this special issue, would undoubtedly open new perspectives and applications.

Keywords Systemic autoimmune diseases · Flow cytometry · B cells · T cells · Monocytes

Introduction

Originally referred to as "micro-fluorimetry" in the 1960s, the ancestor of flow cytometry was able to measure the properties of individual cells (e.g., size, granularity, autofe.luorescence) in suspension following laser illumination. Next, with the development of monoclonal antibodies (mAb), which bound to specific cell surface markers referred to as clusters of differentiation (CD), protocols for intracellular staining, and the discovery of a large panel of fluorescent dyes, new applications were proposed for basic immunological research and clinical biological diagnostics.

Regarding diseases of the immune system, flow cytometry can be used for assessing the immune status of patients with multiple applications ranging from primary immune deficiency to acquired deficiency such as peripheral blood $CD4^+$ T cell counts and CD4/CD8 ratio determination after human immunodeficiency virus (HIV) infection. Another emerging use of flow cytometry is the determination of mononuclear subsets in systemic autoimmune diseases (SAD) to facilitate the diagnosis and to permit follow-up of patients. However, and because of methodological and practical limitations, flow cytometry development in SADs remains limited, but the situation is evolving as presented in this special issue.

✉ Yves Renaudineau
yves.renaudineau@univ-brest.fr

1 Laboratory of Immunology and Immunotherapy, CHU Morvan, Brest, France

2 U1227, Université de Brest, INSERM, LabEx IGO, Cancéropole Grand Ouest, Brest, France

Differentiation Markers

The major subsets of T cells present in peripheral blood mononuclear cells (PBMC) can be defined by the expression of CD4 and CD8, together with CD45RA and CD27 (or CCR7 if using clone 150,503) for distinguishing naive, central memory, effector memory, and effector $CD4^+$ and $CD8^+$ T cells [1]. With the addition of activation markers, such as CD38, CD69, and HLA-DR, it is possible to define activated subsets for each cell type. For Treg, cell surface staining using CD127 or intracellular staining with forkhead box p3 (Foxp3) is recommended.

Initially, and according to the expression of IgD and CD38 and by analogy with tonsillar B cells, peripheral blood B cells were classified into five groups (Bm1-Bm5) corresponding to naïve B cells (Bm1: IgD^+CD38^-), naïve activated B cells (Bm2 $IgD^+CD38^{+/++}$), germinal center B cells (Bm3/4: IgD-$CD38^+$), and memory B cells (Bm5: IgD^-CD38^-) [2]. As memory B cells have emerged as a key subset in SADs, CD27 is actually preferred instead of CD38 to define naïve B cells (IgD^+ $CD27^-$), unswitched memory B cells (IgD^+ $CD27^+$), switched memory B cells (IgD^- $CD27^+$), and double-negative memory B cells (IgD^- $CD27^-$) [3]. In addition to these major subsets, differences may involve B cell precursors, and CD24 associated with CD38 define immature "transitional" B cells ($CD24^{high}CD38^{high}$). The B1 cells, characterized by the production of natural autoantibodies, can be distinguished from B2 cells by using two markers, CD5 and CD45RA [4–6]. On the other side of the B cell spectrum, CD38 or CD138 can define circulating plasmablasts.

Based on the markers CD16 and CD56, natural killer (NK) cells can be subdivided into two major subsets with on the one hand, the NK precursors $CD16^+CD56^{high}$ and, on the other hand, the active NK cells $CD16^+CD56^{low}$. Monocytes may also reveal substantial heterogeneity, but currently two major subsets are widely recognized: classical monocytes (which are $CD14^{high}$ $CD16^-$) and non-classical monocytes (which are $CD14^{low}$ $CD16^{high}$). Within cells negative for T cells ($CD3^+$), B cells ($CD19/CD20^+$), and monocytes ($CD14^+$), dendritic cells (DC) can be identified as $HLA\text{-}DR^{high}$ and subdivided into myeloid DC ($CD11c^+$ cells) and the plasmacytoid DC ($CD123^+$ cells).

Intracellular Markers

Intracellular markers for these cell types are of course informative, since testing intracellular phosphoproteins, cytokines or components of the calcium pathway can provide clues to cluster patients according to these parameters. According to Taher et al. [7], defects in intracellular signaling can lead to B lymphocyte hyperactivity, autoantibody production, SAD development and, for some patients, resistance to treatment. Such modifications can be tested by exploring (i) the B cell receptor (BCR; IgM/D/G and A) and its positive (e.g., CD19, CD2, CD79, CD40) or negative (e.g., CD45, CD5, CD72, CD22, CD32) co-receptors; (ii) BCR proximal kinases (e.g., Lyn, Syk); (iii) downstream kinases (e.g., PI3K, PLC, PKC, MAPK, Akt, mTor) and transcription factors (e.g., NF-kB, NFAT); (iv) innate receptors and signaling (e.g., TLRs, Myd88); and (v) cytokine signaling (e.g., Jak, STAT, cytokines) that define effector B cells and regulatory B cells. Another way to analyze intracellular B cell dysregulation is to test B cell capacity to mobilize intracellular calcium [8]. Important differences are described for this pathway including opposite observations from one disease to another one (e.g., systemic lupus erythematosus versus primary Sjögren's syndrome). Such characterization opens new opportunities for classification and therapeutic.

Technical Considerations for Clinical Trials

Conducting clinical projects using flow cytometry is challenging and requires control of several factors [9].

Sample handling Staining fresh whole blood (<24-h delay) or purified PBMC using a density gradient procedure (e.g., ficoll-hypaque) is the gold standard but not always available when conducting a large clinical trial, or not suitable when conducting a longitudinal study. For the later cases, PBMC cryopreservation is an alternative but with important limitations that have to be known such as rare cell subset depletion (DC, plasmablasts), decreased cell viability and functions, and impact on the expression levels of different markers (e.g., CD62L). To circumvent such limitations, whole blood samples or purified PBMCs can be fixed but with an impact on staining. Both frozen and fixed samples could be analyzed in a batch manner in each center or, better, in a central laboratory.

Reagents The definition of the antibody cocktail is crucial as the choice of the target, antibody clones, antibody titers, and fluorochrome associations can have major influences on the results as well as pipetting variations and fluorochrome degradation. The recent development of lyse-no-wash-no-centrifuge protocols and ready to use tubes with lyophilized reagents can greatly decrease variability and should be the preferred approach whenever possible.

Instrument setup Important differences in performance exist between the large panel of instruments are available from various manufacturers but also for the same instrument due to configuration differences that are related to the laser, filter options, voltage gains, and maintenance. The utilization of stained antibody capture standardized beads and the use of consensus biological controls can be helpful to overcome these difficulties.

Data analysis This is one of the largest sources of variations in flow cytometry due to multiple procedures for analysis and multiple software packages. Therefore, centralized analysis and utilization of automated gating algorithms represent the easiest way to reduce such variability, whereas choices regarding sample handing, reagents, and instruments are irrevocable.

Immunophenotype in Systemic Autoimmune Diseases

As presented by Carvajal-Alegria et al. [10], anomalies of mononuclear subset distribution vary from one SAD to another one. Consequently, such observations have provided the impetus to propose novel diagnostic tools based on the immunophenotype results [11], and some teams have tested integration of these results into the SADs classification criteria, but to date, none of these markers change the well-established diagnostic performance of the reference criteria. This situation is likely to evolve in the next years with the development of new tools for unbiased analysis of multiple mononuclear cell subsets as recently proposed, and it may likely be based on the utilization of 34 markers in mass spectrometry [12].

In line with these observations, the integration of lymphocyte phenotype as a biomarker of therapeutic response is limited but has been confirmed by a systematic review conducted by Schreiber et al. [13]. The main limitations highlighted by the authors are related to the lack of reproducibility that may be explained by several parameters such as patient characteristics (e.g., age, sex, disease duration, concomitant, and previous therapies), pharmacological considerations, the time point used to define responder from non-responder, and technical issues as described previously. However, several promising biomarkers are emerging such as memory B cells [14], and effector T cells that produce either IL-17 or either GM-CSF [15].

Future Directions

As performed in the IMI PRECISESADS project [16], the immunological analysis has to be tightly controlled and all parameters, including pre- and post-parameters, fixed at the design step of the clinical trial. This is one of the European Innovative Medicine Initiative (IMI) projects that includes 2500 patients with SAD and is tested with a large multi-OMIC approach including multi-parameter flow cytometry performed simultaneously in 11 different centers, and this unbiased analysis will allow exploration of mononuclear cell profiles for distinct groups of patients [17].

Another way to gain insight into immune dysregulation in SADs is to use SAD-prone mice for assessing complete immunophenotyping. Such a strategy presents several advantages including the capacity to control the genetic background, the environment, and the treatments. These studies can be relatively small, longitudinal, and not restricted to the analysis of the peripheral blood. However, comparison with humans is not always possible but a reasonable degree of convergence is achievable as presented by De Groof et al. [18].

Finally, flow cytometry is under exponential development, which involves new technical propositions coupled with important progress in data analysis. With the emergence of new cytometric technologies (e.g., mass spectrometry), it is now possible to detect up to 50 protein markers at single-cell resolution. Then, a specific cellular signature can be tested in a complex mixture of cells as observed in PBMC or in infiltrated tissues after mechanical or enzymatic separation. In addition, with this approach, rare but important cellular subsets such as DC can be integrated and their activation status tested [19].

Conclusions

Flow cytometry represents the tool of choice for the analysis of the immune system phenotype and function by testing multiple parameters on many individual cells. Today, the analysis is limited but, with the development of standardized operating procedures and next generation technologies, it is predicted that the analysis of mononuclear subset partitioning will find its place in the development of future personalized medicine. Finally, we hope that you will enjoy this special issue and thank the authors for their contributions.

Acknowledgements The author expresses gratitude for editorial assistance to Dr. W.H. Brooks (University of South Florida, FL, USA), and to Simone Forest and Geneviève Michel for their help in typing the paper.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Funding There is no funding source.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Simonin L, Pasquier E, Leroyer C, Cornec D, Lemerle J, Bendaoud B, Hillion S, Pers J-O, Couturaud F, Renaudineau Y (2016) Lymphocyte disturbances in primary antiphospholipid syndrome and application to venous thromboembolism follow-up. Clinical reviews in allergy & immunology:1–14. doi:10.1007/s12016-016-8568-1
2. Nouel A, Segalen I, Jamin C, Doucet L, Caillard S, Renaudineau Y, Pers JO, Le Meur Y, Hillion S (2014) B cells display an abnormal distribution and an impaired suppressive function in patients with

chronic antibody-mediated rejection. Kidney Int 85(3):590–599. doi:10.1038/ki.2013.457

3. Gazeau P, Devauchelle-Pensec V, Pochard P, Pers JO, Saraux A, Renaudineau Y, Cornec D (2016) Abatacept efficacy in rheumatoid arthritis is dependent upon baseline blood B-cell levels. Rheumatology (Oxford) 55(6):1138–1140. doi:10.1093/rheumatology/kev417
4. Renaudineau Y, Hillion S, Saraux A, Mageed RA, Youinou P (2005) An alternative exon 1 of the CD5 gene regulates CD5 expression in human B lymphocytes. Blood 106(8):2781–2789. doi:10.1182/blood-2005-02-0597
5. Renaudineau Y, Vallet S, Le Dantec C, Hillion S, Saraux A, Youinou P (2005) Characterization of the human CD5 endogenous retrovirus-E in B lymphocytes. Genes Immun 6(8):663–671. doi:10.1038/sj.gene.6364253
6. Renaudineau Y, Bariller E, Pers JO (2014) B1- and CD5-Positive B Cells. In: eLS. John Wiley & Sons, Ltd. doi:10.1002/9780470015902.a0024242
7. Taher TE, Bystrom J, Ong VH, Isenberg D, Y. R, Abraham DJ, Mageed R (2017) Intracellular B lymphocyte signalling and the regulation of humoral immunity and autoimmunity. Clin Rev Allergy Immunol. doi:10.1007/s12016-017-8609-4
8. Hemon P, Renaudineau Y, Debant M, Le Goux N, Mukherjee S, Brooks W, Mignen O (2017) Calcium signaling: from normal B cell development to tolerance breakdown and autoimmunity. Clinic Rev Allerg Immunol. doi:10.1007/s12016-017-8607-6
9. Maecker HT, McCoy JP, Nussenblatt R (2012) Standardizing immunophenotyping for the human immunology project. Nat Rev Immunol 12(3):191–200. doi:10.1038/nri3158
10. Carvajal Alegria G, Gazeau P, Hillion S, Daïen CI, Cornec D (2017) Could lymphocyte profiling be useful to diagnose systemic autoimmune diseases? Clin Rev Allergy Immunol. doi:10.1007/s12016-017-8608-5
11. Cornec D, Saraux A, Cochener B, Pers JO, Jousse-Joulin S, Renaudineau Y, Marhadour T, Devauchelle-Pensec V (2014) Level of agreement between 2002 American-European Consensus Group and 2012 American College of Rheumatology classification criteria for Sjogren's syndrome and reasons for discrepancies. Arthritis research & therapy 16(2):R74. doi:10.1186/ar4514
12. Mingueneau M, Boudaoud S, Haskett S, Reynolds TL, Nocturne G, Norton E, Zhang X, Constant M, Park D, Wang W, Lazure T, Le Pajolec C, Ergun A, Mariette X (2016) Cytometry by time-of-flight immunophenotyping identifies a blood Sjogren's signature correlating with disease activity and glandular inflammation. J Allergy Clin Immunol 137(6):1809–1821. **e1812**. doi:10.1016/j.jaci.2016.01.024
13. Schreiber K, Nocturne G, Cornec D, Daïen CI (in press) Lymphocytes as biomarkers of therapeutic response in rheumatic autoimmune diseases, is it a realistic goal? Clin Rev Allergy Immunol
14. Gazeau P, Alegria GC, Devauchelle Pensec V, Lemerle J, Bendaoud B, Saraux A, Cornec D, Y. R (in press) Switched memory B cells influence response to abatacept in rheumatoid arthritis. Clin Rev Allergy Immunol
15. Bystrom J, Clanchy FI, Taher TE, Al-Bogami M, Mohamed H, Alzabin S, Mangat P, Jawad A, R.O. W, Mageed R (2017) Response to treatment with TNF-alpha inhibitors in rheumatoid arthritis is associated with high levels of GM-CSF and GM-CSF$^+$ T lymphocytes. Clin Rev Allergy Immunol. 10.1007/s12016-017-8610-y
16. Jamin C, Le Lann L, Alvarez-Errico D, Barbarroja N, Cantaert T, Ducreux J, Dufour AM, Gerl V, Kniesch K, Neves E, Trombetta E, Alarcon-Riquelme M, Maranon C, Pers JO (2016) Multi-center harmonization of flow cytometers in the context of the European "PRECISESADS" project. Autoimmun Rev 15(11):1038–1045. doi:10.1016/j.autrev.2016.07.034
17. Hofmann-Apitius M, Alarcon-Riquelme ME, Chamberlain C, McHale D (2015) Towards the taxonomy of human disease. Nat Rev Drug Discov 14(2):75–76. doi:10.1038/nrd4537
18. De Groof A, Hemon P, Mignen O, Pers JO, Wakeland W, Y. R, Lauwerys BR (2017) Dysregulated lymphoid cell populations in mouse models of systemic lupus erythematosus. Clin RevAllergy Immunol. doi:10.1007/s12016-017-8605-8
19. Morell M, Varela N, Maranon C (2017) Myeloid populations are altered in systemic autoimmune diseases. Clin Rev Allergy Immunol. doi:10.1007/s12016-017-8606-7