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ORIGINAL PAPER

High-Sensitivity Immunofluorescence Staining: A Comparison of the Liposome Procedure and the FASER Technique on mGR Detection

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Abstract Flow cytometry has become a widely-used and powerful tool for the characterization of cells according to their expression of specific proteins. However, sensitivity of this method is still limited since conventionally labeled antibodies can be conjugated with at maximum 1–10 dye molecules. This fact resulted in the need to develop new techniques in order to identify molecules which are expressed in very low but functionally relevant amounts. In the past, we have successfully used a liposome-based high-sensitivity immunofluorescence technique to measure the expression of low abundant membrane bound glucocorticoid receptors (mGR) on different cell types. The use of this technique allows the detection of as few as 50–100 antigen molecules per cell which is due to a 100-fold to 1000-fold increase in fluorescence signal intensity compared with conventional methods. The higher sensitivity is achieved since thousands of dye molecules can be enclosed in liposomes. Another modern high-sensitivity immunofluorescence staining method is the purchasable Fluorescence Amplification by Sequential Employment of Reagents (FASER) procedure. Here, we aimed at comparing sensitivity and specificity of these two techniques for the detection of the mGR. Our data demonstrate the FASER technique to be more sensitive and also more specific for the detection of mGR as compared to the liposome technique. However, both methods have advantages and disadvantages which are discussed in detail.

Keywords Flow cytometry · High-sensitivity · Liposomes · FASER · mGR

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Introduction

In the field of recent applied research, it would be unimaginable to miss the immunofluorescence-based analysis as a widely-used and powerful tool for the characterization of cells according to the expression of specific proteins. Moreover, due to recent improvements regarding the sensitivity of this technology, more and more molecules which are expressed in very low but functionally relevant amounts can be detected. The conventional staining method is limited to a minimum of several thousand antigens per cell necessary for a distinct discrimination between positive and negative cells due to the low number of fluorochromes that can be conjugated to one staining antibody [1–5]. One important approach to detect functionally important molecules (e.g. many cytokine or hormone receptors [6]), which are expressed in low amounts at the cell surface, was the development of magnetofluorescent liposomes to increase the sensitivity of immunofluorescence [1]. We were able to show that antibody-conjugated magnetofluorescent

liposomes can detect as few as 50–100 antigen molecules per cell because of an increase in fluorescence signal intensity by about 100-fold to 1000-fold compared with conventional methods [1, 5, 6]. With the help of this technique, Assenmacher et al. have characterized the expression of the surface-bound forms of interleukin (IL)-10 and interferon (IFN)-γ [7]. In another study, surrogate light chain expression during B cell lineage differentiation was examined by the liposome based enhanced immunofluorescence method in murine and human pro-B and pre-B cell lines as well as in primary bone marrow cells [8]. The enhancement of the fluorescence signal with the liposome technique has also been used for the phenotypical and functional characterization of different antigen-presenting cells which present specific peptides in physiological relevant quantities [9, 10]. One example is the presentation of major histocompatibility complex (MHC) class II/peptide-complexes on the surface of antigen-presenting cells (e.g. dendritic cells, B lymphocytes and macrophages) to naive T cells, which is an important step for the induction of an adaptive immune response as well as for the generation of antigen-specific tolerance [11–16]. The analysis of the MHC class II/peptide-complexes was limited due to the cross reactivity and low sensitivity of commercially available monoclonal antibodies [17–20] and due to the low copy number (10 times less than the detection limit of classical immunofluorescence [6]) of the presented peptides [21, 22]. *Kunkel* et al. demonstrated that magnetofluorescent liposomes allow the detection and the magnetic enrichment of antigen-presenting cells at 100- to 1000-fold lower peptide concentrations compared to conventional methods [9]. A further important experiment with use of magnetofluorescent liposomes was the identification of a special subpopulation of endothelial progenitor cells derived from peripheral blood, the CD14+ $CD34^{low}$ cells [23].

A few years ago, our group identified low abundant membrane bound glucocorticoid receptors (mGR) to be expressed on human peripheral blood mononuclear cells using highly sensitive immunofluorescent staining via liposomes [1, 24–26]. Furthermore, stimulation with LPS showed an increase in the frequency of mGR positive monocytes, which can be prevented with brefeldin A, an inhibitor of the secretory pathway [24]. Using the liposome technique, we were able to demonstrate in patients with rheumatic and other inflammatory diseases that the frequency of mGR-positive monocytes is dependent on disease, activity of the disease and/or glucocorticoid treatment [24, 27–29]. Our most recent study revealed that the functional active human mGR and the cytosolic GR are encoded by the same gene [30]. These interesting results would not have been achieved without the highly sensitive immunofluorescent staining via liposomes. Here we aimed at comparing the liposome technique we have previously used with the commercially available and more standardized FASER-APC technique in order to determine sensitivity and specificity.

Materials and Methods

Antibodies and Reagents Lipopolysaccharide (LPS), brefeldin A and propidium iodide (PI) were purchased from Sigma-Aldrich. For flow cytometry, the mouse monoclonal anti-human antibodies anti-CD14 (clone TM1, from the Deutsches Rheumaforschungszentrum), anti-CD4 (clone TT1, from the Deutsches Rheumaforschungszentrum) and anti-GR (5E4), from Timea Berki [31] were used. The anti-digoxigenin/anti-Biotin matrix as well as the FASER kit were purchased from Miltenyi Biotec. The blocking peptide APTEK26, a fragment of the GR and target of the anti-GR, was purchased from GenScript Corporation.

Cell Culture HEK 293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich), 100 units/ml penicillin G, 100 μg/ml streptomycin (both from PAA Laboratories), and 50 μM β-mercaptoethanol (Sigma-Aldrich).

Isolation of Human Primary CD4+ T Cells and CD14+ Monocytes Venous blood (obtained from healthy donors) was collected in heparinized tubes. Ethics committee approval was secured (Ethics Committee of Charité University Hospital, Berlin, Germany) and informed consent from each donor was obtained. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation using the Ficoll-Paque PLUS technique (Amersham Biosciences). CD4+ T cells or CD14+ monocytes were enriched up to 99 % purity and >95 % viability (data not shown) by magnetic-activated cell sorting, using anti human CD4- or CD14-conjugated magnetic beads (Miltenyi Biotec).

Culture of HEK 293 T Cells and Human Primary Cells and Stimulation of CD14+ Monocytes HEK 293 T cells were cultured at $2*10^6$ cells/ml in DMEM for 24 h with 5 μg/ml brefeldin A. Human T cells and monocytes were cultured at $2*10^6$ cells/ml in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated FCS, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. Monocytes were incubated for 24 h in a 24-well plate with 2 μg/ml LPS. Control cells (HEK 293 T cells or monocytes) were resuspended in the respective media and incubated without LPS or brefeldin A in a humidified incubator at 37 °C (18 % O_2/5 % CO_2).

Flow Cytometric Analysis

Liposome Technique High-sensitivity immunofluorescence staining was used for mGR detection, as described previously [1]. Briefly, anti-GR monoclonal antibody (clone 5E4) was conjugated to digoxigenin and used for staining,

followed by incubation with anti-digoxigenin/anti-Biotin matrix, and subsequently biotinylated magnetofluorescent Cy5 liposomes. For specificity controls, cells were incubated (i) without anti-GR-Dig antibody and (ii) with a 50–100 fold excess of either unlabeled anti-GR antibody or specific peptide (APTEK26) [31] prior to staining with the anti-GR-Dig conjugate. The frequency of mGR positive cells was calculated from the positive sample (*staining*) by subtracting the background signals obtained by blocking (*block*) [28].

FASER-APC Technique Fluorescence intensity is amplified via FASER Kit-APC by the sequential addition of the reagents as described by the manufacturer (Miltenyi Biotec). Briefly, cells were labeled with an APC-conjugated anti-GR monoclonal antibody (clone 5E4) and then incubated with FcR blocking reagent and APC-Activator reagent for 10 min in the dark at 4 °C. After incubation, cells were washed and immediately analyzed. The procedure can be repeated to increase the fluorescence intensity even to a greater extent.

For functional analysis, isolated T cells and monocytes were identified using anti-human CD4 or CD14 antibodies.

Dead/apoptotic cells were excluded by adding PI before cell acquisition, using a BD FACS Calibur flow cytometer. The acquired data were analyzed using FlowJo 7.6.1 software.

Results

Our first aim was to determine the adequate liposome or antibody concentration and the number of repetitions required for the addition of the FASER reagents.

The mGR is Detectable with the Liposome Technique and the FASER-APC Procedure

For an adequate comparison of both methods, we need a model system for which the extent of mGR expression is already known and has been verified by one of the methods, and in which expression of mGR is stable. Our "gold standard" is represented by human embryonic kidney 293 T (HEK 293 T cells) cells, which express the mGR at a high level as it had been shown via the liposome technique. As this transformed cell line originates from a single cell type, we can assume that every cell expresses the mGR and thus theoretically 100 % of the cells would be stained equally. It is known, however, that mGR expression is dependent on the cell cycle [32], therefore we always used cells of at least 70 % confluence to achieve optimal expression.

As mGR negative control we used human primary T cells. These cells are known to own a functional active membrane-linked glucocorticoid receptor within the T cell receptor (TCR) complex [33–37]. Due to the fact that the required epitope is not accessible for our monoclonal GR antibody clone 5E4, this membrane-linked GR is not detectable with the liposome technique. Hence, we can assume that equally for all T cells mGR detection is nearly zero.

These two cell types were used to set up both staining methods. As a rule, every new batch of liposomes has to be titrated carefully (Fig. 1). The main aim is to detect as many mGR expressing cells as possible while keeping the concurrent unspecific staining as low as possible, the latter being controlled by blocking with either unlabeled anti-GR antibody in excess or with the specific peptide (APTEK26). Here, we tested with the help of HEK 293 T cells three different liposome dilutions (1:5000; 1:7500; 1:10000) (Fig. 1a). The typical result achieved by this method is a bimodal distribution of the fluorescence intensity, which can be attributed to a mGR positive and a mGR negative population of the cells. By increasing the dilution of liposomes, the frequency of mGR positive cells but also the quantity of unspecific binding is reduced. In our example, the optimal result with 73.3 % effective specific staining (calculated by subtracting the frequency of mGR positive cells of the block from the frequency of mGR positive cells of the staining) is achieved with the lowest dilution (1:5000) of the liposomes. As a control, HEK 293 T cells were stained without liposome enhancement with the help of *conventionally* labeled anti-GR antibody, which led to an effective staining of 2.5 % mGR positive cells only (Fig. 1b). Liposome staining of human T cells, used as mGR negative control, resulted in a frequency of 1 % mGR positive cells (Fig. 1c).

A similar approach was performed for the setup of the FASER-APC procedure. We found that the whole population showed an increase in fluorescence intensity (observable as shift to the right) and becomes mGR positive instead of the bimodal distribution, which is typically found with the liposome procedure. The anti-GR APC antibody was tested in three different dilutions (1:100, 1:200; 1:300 which is in accordance with the concentrations of 3.6, 1.8, and 1.2 μg/ml) (Fig. 2a). Due to the unimodal distribution of the fluorescence intensity, we conclude that a shift of the whole population results in 100 % mGR positive cells. Therefore, we also analyzed the mean fluorescence intensity (geometric mean, given in the histogram). Regarding both, the percentage of positive cells and the mean fluorescence intensity, we similarly found the lowest dilution (which means the highest concentration) to be most effective.

As a control, the staining procedure was done with *conventionally* labeled anti-GR antibody (without adding the FASER enhancement reagents), which led to mean fluorescence intensity about 7.30 (in accordance to mean values between 20 and 25 with FASER procedure) (Fig. 2b). FASER-APC staining of human T cells, used as mGR negative control, resulted in weak mean fluorescence intensity, which is approximately the value found for control and/or block (Fig. 2c).

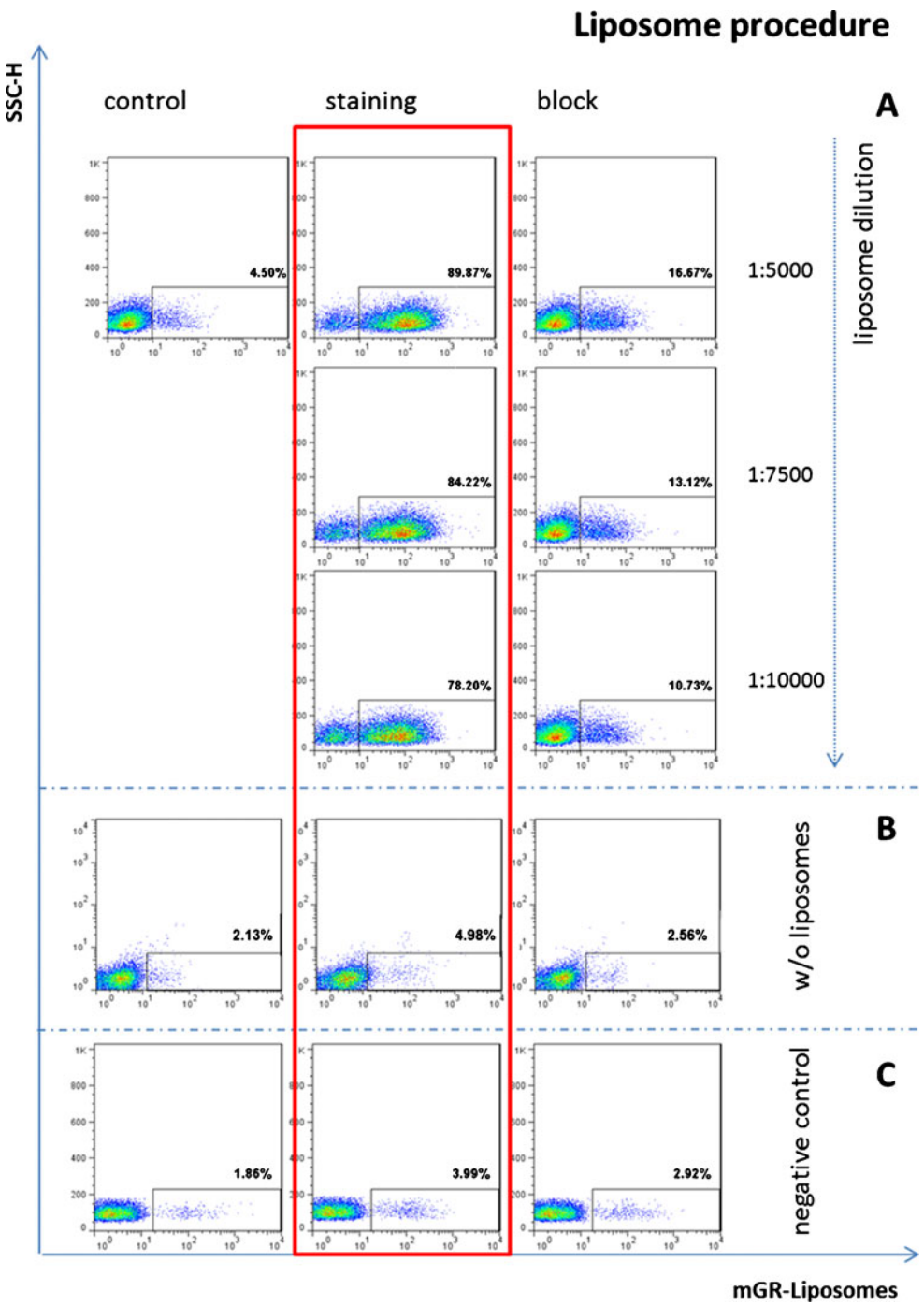

Fig. 1 Liposome procedure—Titration of liposomes and control staining. Using our "gold standard" HEK 293 T cell line, every new batch of liposomes has to be titrated carefully (**a**). Control staining was performed without anti-GR-Dig antibody and block was realized by adding unlabeled antibody in 50-100fold excess. Specificity controls were performed using HEK 293 T cells without adding liposomes but *conventionally* labeled anti-GR antibody (**b**) and human CD4 positive T cells (negative control) with adding liposomes (**c**). Liposome procedure results in a bimodal distribution of clearly separated unstained and stained cells, whereas quantitative analysis was performed using gates on positive stained cells (staining; *red border*)

The FASER-APC Procedure is More Sensitive and More Specific Than the Liposome Technique

Obviously, mGR can be detected by both methods, but which one is more suitable? The most often used parameters to measure the performance of test systems are sensitivity and specificity. While *sensitivity* measures the proportion of actually positives which are correctly identified as such, *specificity* measures the proportion of negatives which are correctly identified. Due to the fact, that one main difference between both methods is reflected by a bimodal and a unimodal distribution of the fluorescence intensity of stained cells, an appraisal of the results is challenging. For comparison of the methods and the following calculations we considered the frequency of mGR positive cells only (and not the mean fluorescence intensity), as it can be determined by both methods regardless of the distribution of the fluorescence intensity.

In our case, HEK 293 T cells are suitable for calculation of the sensitivity whereas human primary T cells

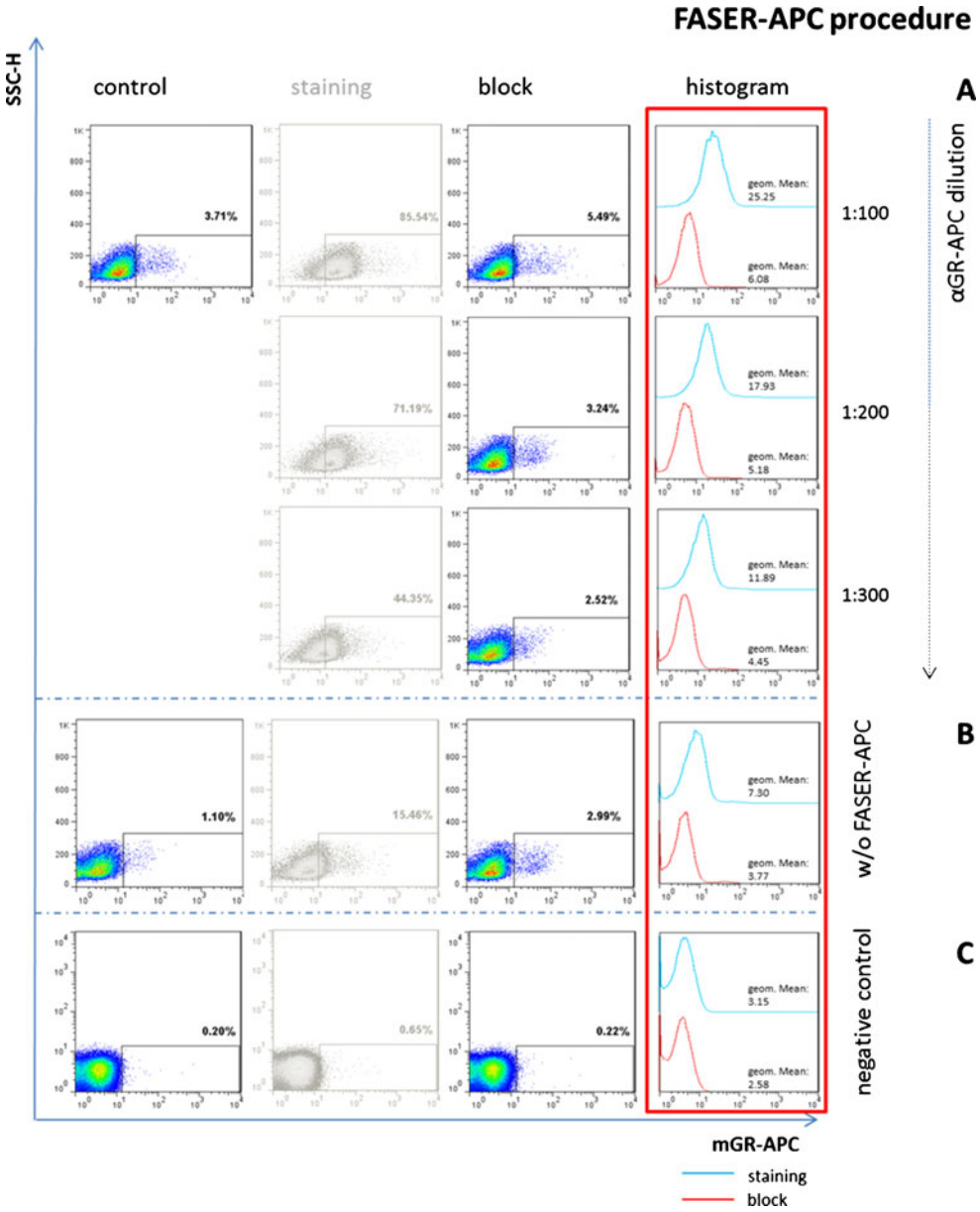

Fig. 2 FASER-APC procedure—Titration of antiGR-APC antibody and control staining. Using the HEK 293 T cell line, the anti-GR-APC antibody was titrated carefully (**a**). Control staining was performed without anti-GR-APC antibody and block was realized by adding unlabeled antibody in 50-100fold excess. Specificity controls were performed using HEK 293 T cells without adding FASER-APC reagents but *conventionally* labeled anti-GR antibody (**b**) and human CD4 positive T cells (negative control) with adding FASER enhancer (**c**). FASER-APC procedure results in an unimodal distribution of cells, whereas results are additionally represented and analysed as histograms (*red border*; overlay of staining and block) with the calculated geometric means for all samples (*last column*)

are suitable for calculation of the specificity of both methods. One exemplary calculation, for which both stainings were performed for one cell type, is given in Fig. 3. The test outcome can be positive (predicting that the HEK 293 T cells express the mGR) or negative (predicting that T cells do not express the mGR on their surface). The test results for each measurement may or may not match the estimated status. We can define the following results:

- True positive: mGR positive HEK 293 T cells correctly detected as mGR positive
- False positive: mGR negative T cells incorrectly identified as mGR positive
- True negative: mGR negative T cells correctly detected as mGR negative
- False negative: mGR positive HEK 293 T incorrectly identified as mGR negative

The two parameters were calculated as follows:

- Sensitivity
  = number of true positives/(number of true positives + number of false negatives)
- Specificity
  = number of true negatives/(number of true negatives + number of false positives)

The exemplary calculation resulted in a sensitivity of 73.2 % and a specificity of 96.4 % for the liposome procedure. For the FASER-APC procedure we found a sensitivity of 94.5 % (100 % positive cells reduced about 5.5 % block) and a specificity of 99.1 % for our example (Fig. 3).

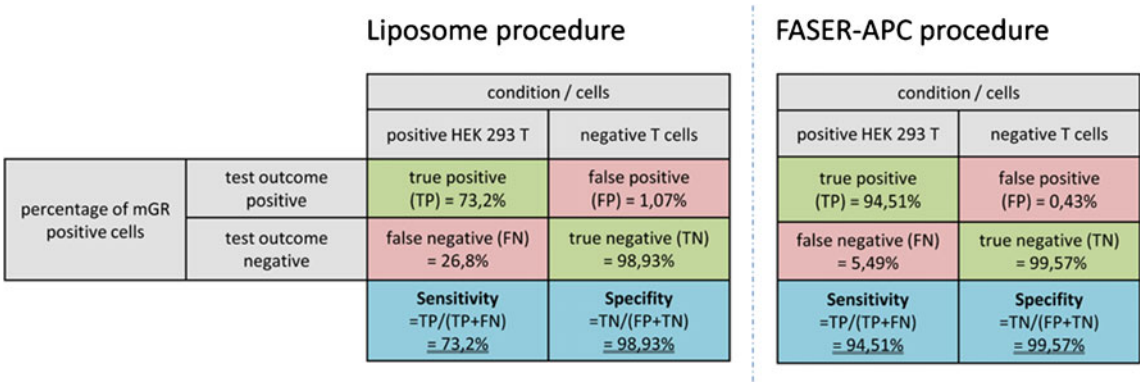

Fig. 3 Comparison of liposome procedure and FASER-APC procedure. Exemplary calculation of sensitivity and specificity of liposome procedure and FASER-APC procedure

Changes of mGR Expression are Also Detectable with the FASER-APC Procedure

We next wanted to assess whether the FASER-APC procedure can also properly detect changes in mGR levels in a given cell type. To this end we can take advantage of the fact that it has been shown that transportation of mGR to the cellular membrane can be prevented with brefeldin A [24], an inhibitor of protein transport to the Golgi complex [38]. Furthermore, we found that immunostimulation with lipopolysaccharide (LPS) increases the percentage of mGR positive monocytes [24].

We here inhibited mGR transport with brefeldin A in HEK 293 T cells and increased the frequency of mGR expression in human monocytes with LPS (Fig. 4). With the liposome technique, we were able to show a decrease in mGR expression in brefeldin A treated HEK 293 T cells from about 64 % positive cells to about 35 % positive cells, which means a reduction by about 50 % (Fig. 4a). With FASER-APC method the mGR transport inhibition resulted in a decrease in mean fluorescence intensity (geometric mean, displayed in the histogram) from 12.12 to 5.02, which is almost the value found for control and/or block (Fig. 4c). Furthermore, we were able to detect an approximately three fold increase (from 7 % mGR positive cells to about 22 % positive cells) of mGR expression in LPS treated monocytes with the liposome technique (Fig. 4b). In comparison, the FASER-APC method resulted in an increase in mean fluorescence intensity (geometric mean, displayed in the histogram) from 11.45 to 15.19 (Fig. 4d).

Discussion

During the last years, flow cytometry has become an important and powerful tool in the field of research and development, foremost in the field of immunology. Due to the detection of specific proteins, it is possible to differentiate between subpopulations within one cell type. The method itself has become more sensitive; hence, we are able to detect molecules which are expressed in very low but functionally relevant amounts. An increase in fluorescence signal intensity about 100-fold to 1000-fold compared to conventional methods [1, 5, 6] with *high-sensitive immunofluorescence staining* was initially realized with the help of magnetofluorescent liposomes. Using this technique, surface-bound forms of interleukin (IL)-10 and interferon (IFN)-γ [7] or MHC class II/peptide-complexes on the surface of antigen-presenting cells were identified. In addition, TCRβ [39] and CD152 [40], which are expressed below, or just at the limit of detection by standard methods could be detected on the surface of T cells with this method. Finally, we were able to detect membrane bound glucocorticoid receptors (mGR) on human peripheral blood mononuclear cells using the liposome technique [24–26]. With technological progress, advancements like the commercialized FASER (Fluorescence Amplification by Sequential Employment of Reagents) technique found its way into the field of flow cytometry.

In the present work, we aimed at clarifying whether the FASER technique is capable of mGR detection, and whether this method is comparable to the liposome technique in regard to sensitivity and specificity.

We would first like to describe both methods, which are based on enhancement of fluorescence intensity, in more detail (Fig. 5). As the name implies, in the liposome procedure the fluorescence signal is increased with the help of well-defined magnetofluorescent liposomes, which contain several thousand cyanine 5 (Cy5) molecules (only 1–10 dye molecules can be conjugated to conventional labeled antibodies!). The liposomes are coupled to biotin, which allows binding to the anti-digoxigenin/anti-Biotin matrix (Fig. 5a). For the detection of the human mGR, which is expressed on the surface of different primary cells as well as on different cell line cells,

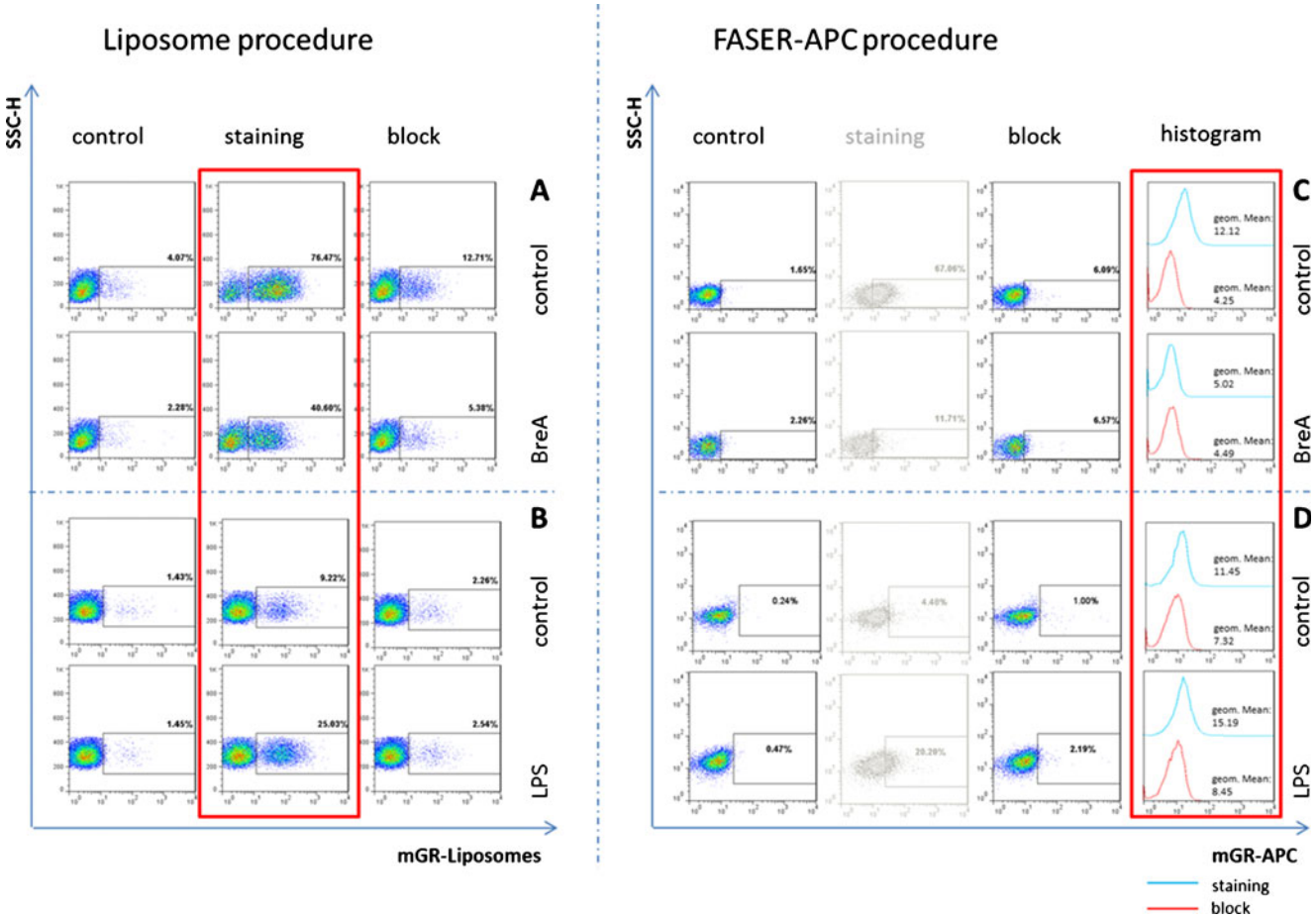

Fig. 4 Inhibition of mGR transport and induction of mGR expression. mGR detection at varying mGR levels via liposome and FASER-APC procedure. Inhibition of mGR transport to the cellular membrane was performed by adding brefeldin A to HEK 293 T cells, an inhibitor of protein transport to the Golgi complex (**a**, **c**). Immunostimulation with lipopolysaccharide (LPS) increases the percentage/fluorescence intensity of mGR positive human monocytes (**b**, **d**). Results achieved by FASER-APC procedure are additionally represented as histograms (overlay of staining and block) with the calculated geometric means for all samples (**c**, **d**; *last column*). The appropriate analysis mode is highlighted with a red border

we used the monoclonal IgG1 antibody anti-GR 5E4 (conjugated to digoxigenin (Dig)) [31] directed against the conserved regulatory sequence of human GR (aa150-176).

The same monoclonal antibody, conjugated to the fluorochrome allophycocyanin (APC), was used for the FASER procedure (Fig. 5b). With the help of this method, the fluorescence intensity is amplified by adding two reagents: The fluorochrome-specific activator (Reagent 1) and the fluorochrome-conjugated enhancer (Reagent 2). In accordance with the manufacturers, the sequential addition of reagents can be performed as often as required.

The following major findings emerged from this study: (i) mGR is detectable with the FASER Kit-APC. (ii) Moreover, the FASER-APC technique is more sensitive and specific compared to the liposome technique; accordingly, (iii) minor changes of mGR expression can also be demonstrated with the FASER technique.

To date, mGR cannot be reliably detected with conventional staining methods, thus an enhancement of the fluorescence signal intensity is essential. One of the first methods to increase the sensitivity of immunofluorescence was based on the liposome technology, which is nowadays a complex and time consuming procedure, especially with regard to the production of the none commercially available liposomes. Hence, this method underlies minor to major variations depending on the liposome charge. Since a more standardized procedure would represent a major advantage, the commercialized FASER kits represent an alternative due to a consistent quality and a standardized production.

Obviously, in this study the FASER technique is more sensitive and more specific (a comparative overview is given in Table 1). Furthermore, due to a single sequential addition of FASER reagents (a second addition of the reagent led to an increase in unspecific binding, data not shown) this method is more time-saving compared to the liposome technique. However, when analyzing the results more in detail, it is evident that there is a major qualitative difference of the staining pattern. The liposome procedure

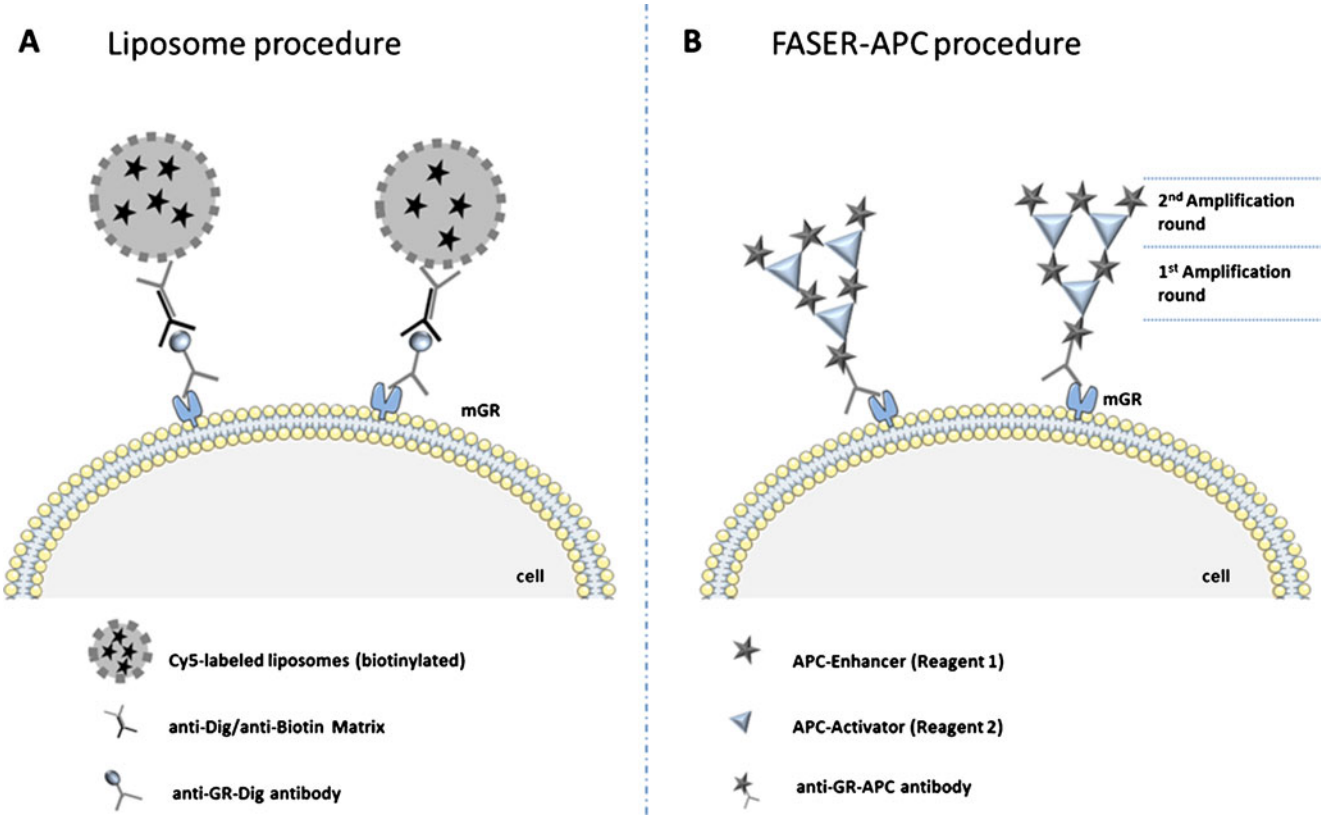

Fig. 5 Enhancement of the fluorescence intensity by liposome and FASER-APC system. Schematic diagrams of the liposome and the FASER-APC staining. The fluorescence signal of the low copy antigen mGR is increased with the help of Cy5-labeled liposomes (**a**). For the FASER procedure the fluorochrome-specific activator (Reagent 1) and the fluorochrome-conjugated enhancer (Reagent 2) are sequentially added (**b**)

typically results in a bimodal distribution of clearly separated unstained and stained cells, suggesting that the target antigen is expressed only by a subset of the cells. For this method it has been described that for antigen densities less than a few hundred molecules the labeling is not quantitative, i.e. at a certain threshold some cells are labeled with liposomes resulting in a clearly detectable signal, whereas other cells are not labeled and appear completely negative.

In contrast, the FASER procedure results in a shift of the whole population, which would indicate that all cells express the antigen albeit at different levels. Due to this unimodal distribution of cells, it is necessary to analyze the mean

Table 1 Major differences between the compared high-sensitivity immunofluorescence staining methods. Differences concerning technical aspects and result-oriented aspects of the liposome method and the FASER-APC procedure are displayed as overview

Points of comparison	High-sensitivity immunofluorescence staining method	
	Liposome procedure	FASER-APC procedure
Technical aspect		
Availableness	Individual production of components	Commercial available kit
Protocol	Depending on individual produced components	Standardized
Handling	Liposome are sensitive	Very easy
Expenditure of time	Very time consuming	About 1 h time saving
Result-oriented aspects		
Detection of mGR	+	+
Sensitivity	+	++
Specificity	+	++
Distribution	Bimodal	Population shift
Fluorescence intensity	+++	+

fluorescence intensity of these samples. Thus the FASER technology results in a more quantitative labeling allowing to determine subtle differences in antigen expression levels although the overall signal intensity is 2 to 3-fold lower compared to the liposomes (we found geometric mean values up to 68; data not shown).

The liposome procedure has already been used to identify the CD14+ $CD34^{low}$ subpopulation of endothelial progenitor cells derived from peripheral blood [23]. In this study, FASER procedure was performed in parallel to liposome staining. Comparing both methods in their study, the detection of CD34 by the conventional flow cytometry was impossible but the expression of CD34 became obvious when using high-sensitivity immunofluorescence staining. Generally, they found similar results for liposome technique and FASER procedure. But interestingly, when analyzing these results in more detail, the differences in the quality of staining can be correlated to the results we found in our study. There is a bimodal distribution (CD34 positive and negative cells) with liposome technique, while FASER procedure results in a shift of the whole population. The frequency of positive cells obtained with both stainings was similar and the FASER method also seemed to be more specific since CD34 negative cells were more frequently negative compared to the liposome treatment (0.3 % vs. 3.8 %).

Conclusion

Since we firstly detected mGR on human peripheral blood mononuclear cells using the liposome technique [24–26], this receptor is a main focus of our research [1, 24, 25]. Unfortunately, the liposome procedure is complex and time consuming, especially with regard to the production of the liposomes, which are not commercially available. For this reason we compared our established method with a new one, the commercialized FASER technique.

Altogether we can summarize, that both methods are suitable for the detection of the low copy protein mGR. The FASER technique can be recommended for detection of low copy antigens, especially since the sequential addition of FASER reagents can be repeated and thus the signal intensity can be increased stronger. A clear distinction between positive and negative cells is practicable with the liposome technique, although experienced flow cytometry users can also handle and analyze the population shift achieved by the FASER method, especially when using the mean fluorescence intensity of the cells. Furthermore, with the increase in sensitivity and specificity of the available methods, some findings should be recapitulated once more to be analyzed and discussed again. One example is given in Fig. 4b and c. LPS stimulated monocytes show an increase in the frequency of mGR-positive cells when stained with liposome procedure. In contrast, the more sensitive FASER method results in a total up-regulation of mGR protein.

Thus, with advancing methods it is possible to up-date our knowledge, including mGR. There are advantages and disadvantages for both methods but eventually everybody has to decide for himself, which method is most appropriate.

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Competing Interest Statement There are no competing interests.

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