



Basophil Activation after Nonsteroidal Anti-inflammatory Drugs Stimulation in Patients with Immediate Hypersensitivity Reactions to These Drugs

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• Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the drugs most frequently involved in allergic reactions of which two main types exist: IgE-mediated and crossintolerance. The diagnosis of crossintolerance reactions is often based on the drug provocation test. The potential value of the basophil activation test (BAT) was evaluated using different basophil markers in the diagnosis of patients with crossintolerance to NSAIDs and cutaneous symptoms. We studied 46 patients with crossintolerance to NSAIDs and 45 tolerant controls. BAT was performed with acetyl salicylic acid, paracetamol, diclofenac, dipyrrone, naproxen, and ibuprofen at four different concentrations using CD193 and CD203c as basophil markers and CD63 as activation marker. We compared BAT results using CD193⁺ or CD193⁺CD203c⁺ for basophil selection and found a significant increase in the stimulation index when using CD193⁺CD203c⁺ in both patients and controls ($P = 0.004$ and $P = 0.017$, respectively). Selection of living cells only produced an increase in basophil stimulation in patients for both CD193⁺ and CD193⁺CD203c⁺ ($P < 0.001$ for both), whereas in controls there was no change with CD193⁺ and a decrease with CD193⁺CD203c⁺ ($P = 0.001$). We found that CD193⁺CD203c⁺ increased the percentage of positive cases in patients and controls when compared with CD193⁺. When excluding dead cells, there was an increase of 21.7% in patients and 10% in controls. These results indicate that using CD193⁺CD203c⁺, excluding dead cells, is the best approach for BAT although this test is not recommended for the diagnosis of patients with crossintolerance to NSAIDs owing to its low sensitivity and specificity. © 2014 International Society for Advancement of Cytometry

• Key terms

hypersensitivity; nonsteroidal anti-inflammatory drugs; idiosyncratic reactions; basophils; CD63; CD193; CD203c

NONSTEROIDAL anti-inflammatory drugs (NSAIDs) are the drugs most frequently involved in immediate or acute hypersensitivity reactions, accounting for 20–25% of patients evaluated in allergy units, with two main mechanisms involved (1–3). One is mediated by drug-specific-IgE antibodies and reactions occur with one NSAID but there is tolerance to other nonchemically related drugs (4,5). These reactions are known as single NSAID induced hypersensitivity reactions and involve NSAIDs such as paracetamol (6), acetyl salicylic acid (ASA) (7), diclofenac (8), ketorolac (9), dipyrrone (10,11), and cyclooxygenase-2 selective inhibitors (COX-2) (12). The other type of reaction is mediated by a pharmacological mechanism in which the inhibition of cyclooxygenase-1 enzyme (COX-1) leads to a decrease in the synthesis of prostaglandin E₂, which in turn hampers the production of sulphidoleukotrienes

(13,14) and increases other mediators responsible for the cutaneous and respiratory manifestations (14,15). These patients react to NSAIDs from different chemical groups and are therefore classified as crossreactive hypersensitivity reactions to NSAID (3).

As no well-validated tests yet exist, the diagnosis of both types of reaction is mainly based on the clinical history and/or a drug provocation test (DPT) (16,17). Over the last 10 years, an *in vitro* test, the basophil activation test (BAT) using flow cytometry technology, has been used to evaluate IgE-mediated hypersensitivity to different drugs such as betalactams (18,19), corticosteroids (20), quinolones (21), and muscle relaxants (22,23). Regarding NSAIDs, in IgE-mediated reactions to dipyrone, BAT has shown a sensitivity of 42.3–54.9% and a specificity ranging from 85.7 to 92% (24,25). Notably, BAT was positive in 38.5% of patients with negative skin tests but even more importantly, BAT was positive in 75% of patients with anaphylaxis and negative skin tests.

As basophils can be activated by both IgE and non-IgE-mediated mechanisms (14,24,25), the BAT could be used to evaluate NIUA; indeed, it has already been used though with contradictory results (26–29). Although some studies have reported successful results (sensitivity from 43 to 58% with specificity from 90 to 100%) (27,28), others have found a sensitivity of 30% with a specificity of 40% (29); differences were also found in a European multicenter study (30). These differences can be attributed to the patients included with apparently better results obtained in those cases with skin symptoms and clearly no response in those with respiratory manifestations (27–30). As it has recently been shown that the most frequent manifestations appearing in hypersensitivity to NSAIDs are cutaneous (without underlying chronic diseases) classified as NSAID-induced urticaria/angioedema (NIUA) (2,4,31), we considered it important to analyze the role of BAT in this group of patients.

On the other hand, controversy exists about the efficacy of the different markers that can be used to select basophils in the BAT. IgE has been classically used as the main marker (32), but high interindividual variations in cell surface expression of the IgE-receptor (FcεRI) (33,34), a downregulation upon IgE-mediated activation (35), and major contamination with monocytes (36) lead to difficulties in basophil gating which could decrease the sensitivity of the test. Other marker is CD193 (CCR3), which has been identified as a stably expressed marker that allows a robust and accurate selection of basophils though it is also expressed on eosinophils (37), and CD203c, which is selectively expressed on basophils (38) and although it is constitutively present on resting basophils it shows upregulation after activation (39,40). The new basophil selection strategies using these markers or their combination could improve the BAT results.

In this study, we performed BAT using two different markers, CD193 and CD203c, for basophil selection, a dead cell marker and CD63 for the determination of basophil activation. We included a wide panel of NSAIDs in a large number of well-characterized patients with NIUA as compared with tolerant subjects.

MATERIALS AND METHODS

Patients and Controls

The study included patients with NIUA including the following symptoms: urticaria, angioedema, urticaria and angioedema (UA), or urticaria and systemic symptoms (USS) diagnosed over a 3-year period (2010–2013). The classification criteria for NIUA included the presence of at least three episodes with three or more different NSAIDs as described earlier (41). If fewer than three NSAIDs were involved, a positive DPT with a different NSAID was required.

Exclusion criteria included patients with chronic urticaria or acute recurrent urticaria not attributed to NSAID intake; pregnant or breastfeeding women; treatment with beta-blockers or angiotensin-converting enzyme inhibitors; contraindications for epinephrine administration; acute infections and/or underlying cardiac, hepatic, or renal disease that contraindicated DPT; psychosomatic disorders. A control group was composed of subjects without chronic or acute recurrent urticaria and with confirmed good tolerance to NSAIDs.

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethics Committees of each participating hospital. All the patients and controls were informed orally about the study and signed the corresponding informed consent.

Drug Provocation Test

We performed a single-blind placebo-controlled DPT beginning with the NSAID with the lowest COX-1 inhibiting capacity, and if negative we continued by administering others with a higher COX-1 inhibiting capacity as follows: etoricoxib, paracetamol, piroxicam, ibuprofen, indomethacin, and ASA. In patients reporting three or more episodes to different non-chemically related NSAIDs and classified as having NIUA from their clinical history, alternative drugs such as paracetamol or COX-2 inhibitors were tested, provided they themselves were not involved in the reactions (2). NSAIDs were administered orally at increasing doses at 90 min intervals as described earlier (2). If cutaneous or respiratory symptoms or hypotension appeared, the procedure was stopped and the symptoms were evaluated and treated.

Basophil Activation by Flow Cytometry

One hundred microliters of heparinized whole blood was aliquoted per test and 20 µL of stimulation buffer and 3 µL of monoclonal antibody CD193-APC (Caltag Laboratories, Burlingame, CA) were added and incubated for 10 min at 37°C in a water bath. After this, 100 µL of the washing solution was added to the negative control tube and 100 µL of fMLP (chemotactic peptide *N*-Formyl-Met-Leu-Phe) or anti-IgE (BD Pharmingen, NJ) to the positive control tube. One hundred microliters of different NSAIDs were added to the different samples. ASA at 1, 0.5, 0.2, and 0.1 mg/mL, diclofenac at 0.3, 0.15, 0.06, and 0.03 mg/mL, naproxen at 1, 0.5, 0.2, and 0.1 mg/mL, dipyrone at 5, 2.5, 0.25, and 0.1 mg/mL, and ibuprofen at 5, 1.5, 0.75, and 0.15 mg/mL (all from Sigma-Aldrich, Saint Louis, MO), and paracetamol (Novartis Farmaceutica

Table 1. Clinical characteristics of the patients included in the study

PAT.	AGE	SEX	DRUG INVOLVED	INTERVAL REACTION– EVALUATION (MONTHS)	INTERVAL DRUG INTAKE– REACTION (MIN)	REACTION	POSITIVE DPT	NEGATIVE DPT
1	33	F	ASA	108	<60	USS	Ibu	Etori, Parac, Pirox
2	49	M	ASA, Dipy, Parac	120	<60	UA		Etori
3	40	F	Ibup	36	<60	USS	Pirox	Etori, Parac
4	75	M	Dicl, Ketor, Dipy	24	<60	URT		Etori, Parac
5	27	M	ASA, Ibup	18	60	ANG	Pirox	Etori, Parac
6	29	M	ASA	5	48	USS	Indom	Etori, Parac
7	38	F	Parac	4	90	USS	Pirox	Etori
8	53	M	Ibup, Dipy	2	90	URT	ASA	Etori, Parac, Pirox
9	31	M	Ibup	36	90	URT	ASA	Etori, Parac, Pirox
10	38	F	Dicl, Ibup	8	120	URT	ASA	Etori, Parac, Pirox
11	50	M	ASA, Ibup, Dicl, Dipy	12	<60	ANG	Parac	Etori
12	34	M	Dipy	2	<60	USS	Parac	Etori
13	20	F	Dicl, Ibup	13	<60	URT	ASA	Etori
14	49	M	Parac	2	90	URT	Pirox	Etori, Parac,
15	33	M	ASA, Ibup	120	<60	URT	Pirox	Etori, Parac,
16	67	F	Dicl, Ibup, Parac	6	<60	URT	Etori	
17	32	F	ASA, Dicl, Ibup	3	<60	USS	Parac	Etori
18	55	F	ASA, Dicl, Ibup	6	30	USS		Parac
19	79	M	Dicl	12	<60	USS	ASA	Etori, Parac, Pirox
20	33	F	Ibup, Dipy	12	<60	URT	Pirox	Etori, Parac
21	33	F	Dipy	60	5	USS	ASA	Etori, Parac
22	41	F	Dipy	12	60–120	URT	Ibup	Etori, Parac, Pirox
23	20	F	Ibup, Dipy, Ketor, Parac	12	10	ANG		Etori
24	34	M	ASA, Ibup, Parac	2	<60	URT	Etori	
25	27	F	Dipy, Nap	8	5	USS	Pirox	Etori, Parac
26	49	M	ASA, Ketor	7	<60	URT	Pirox	Etori, Parac
27	46	F	Dipy	4	<60	URT	Etori	
28	46	F	Dipy	4	<60	URT	Etori	
29	44	M	ASA, Ibup	84	<60	URT	Pirox	Etori, Parac
30	25	M	ASA, Ibup, Dipy	24	120	ANG		Etori, Parac, Pirox
31	22	F	Dicl	8	120	URT	Ibup	Etori
32	42	M	ASA	25	<60	URT	Ibup	Etori, Parac, Pirox
33	30	F	Dipy	4	<60	URT	Pirox	Etori, Parac
34	19	F	Ibup, Nap	4	60–120	URT	ASA	Etori, Parac, Pirox
35	51	M	ASA, Parac	36	60–120	URT	Pirox	Etori
36	27	F	ASA, Dipy	16	<60	USS	Ibup	Etori, Parac, Pirox
37	28	F	Dipy	96	5	USS	Ibup	Etori, Parac, Pirox
38	45	F	Ibup	117	<60	URT	Pirox	Etori, Parac
39	45	F	Ibup	32	90	URT	Pirox	Etori, Parac
40	67	F	ASA, Dipy	36	<60	URT	Ibup	Etori, Parac, Pirox
41	44	M	Ibup	12	90	ANG	Pirox	Etori, Parac
42	48	F	ASA, Dipy	4	90	URT	Ibup	Etori, Parac, Pirox
43	66	F	Dicl, Dipy	21	90	URT	Pirox	Etori, Parac
44	44	F	Ibup, Pirox	48	80	URT	ASA	Etori, Parac
45	24	M	Dicl, Dipy	5	60–120	URT	Ibup	Etori, Parac
46	32	M	ASA	2	90	UA	Indom	Etori, Parac, Pirox

Abbreviations: ASA, acetyl salicylic acid; Dicl, diclofenac; Napr, naproxen; Parac, paracetamol; Dipy, dipyrone; Ibup, ibuprofen; Pirox, piroxicam; Ketor, ketorolac; Etori, Etoricoxib; Melox, meloxicam; Celec, Celecoxib; Indom, indomethacin; URT, urticaria; ANG, angioedema; UA, urticaria plus angioedema; USS, urticaria plus systemic symptoms.

SA, Barcelona, Spain) at 5, 1, 0.5, and 0.1 mg/mL. Concentrations higher than those described above are cytotoxic and lower concentrations decrease sensitivity. The samples were

incubated for 30 min at 37°C in a water bath. The degranulation was stopped by incubating the samples on ice for 5 min and then 3 µL of monoclonal antibodies, anti-CD63-FITC,

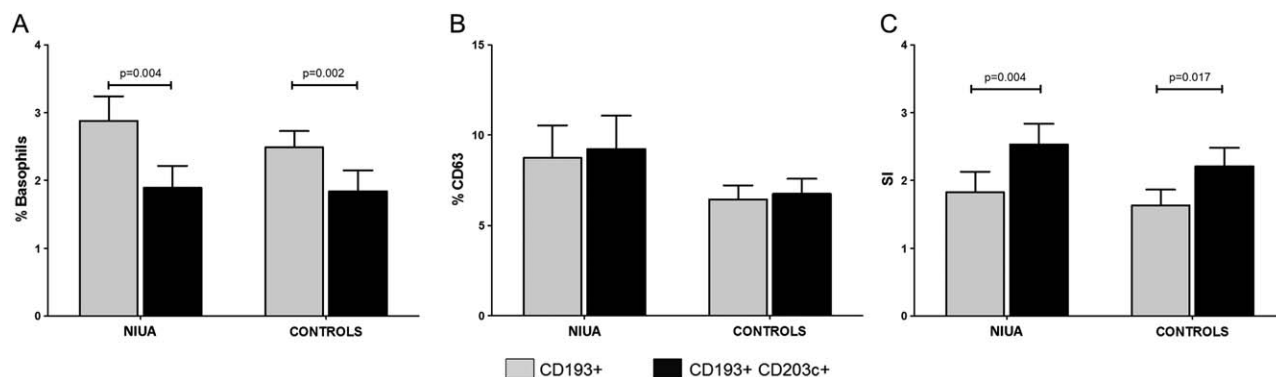


Figure 1. Percentage of basophils recovered (A), percentage of CD63 expressing cells (B), and SI value (C) using two different selection strategies, CD193 alone or with CD203c in NIUA and control group. (Bars represent the mean of 20 determinations and the SEM in 46 CI patients and 45 tolerant subjects).

CD203c-PE (Caltag Laboratories, Burlingame, CA), and Live/Dead Fixable FarRed Dead Cell Stain Kit (Invitrogen, Paisley, United Kingdom), respectively, were added to each tube. After this, 2 mL of prewarmed lysing solution was added and incubated for 10 min at room temperature. After centrifuging and washing, the cells were analyzed in a FACSCalibur flow cytometer (Becton-Dickinson Bioscience, San Jose, CA) by acquiring at least 500–1,000 basophils per sample. Basophils were selected using two different strategies, taking into consideration all the CD193⁺ cells or only the CD193⁺CD203c⁺ cells. In both cases, the results were analyzed with and without the dead cells (Supplemental Figure 1). Results were expressed as the percentage of CD63 and as the stimulation index (SI), calculated as the ratio between the percentage of degranulated basophils (CD63⁺ cells) with different haptens and the negative control. The percentage of basophils activated after contact with the antigen was required to be $\geq 5\%$ as described previously (25).

Statistical Analysis

Comparisons of quantitative variables without a normal distribution were done by the Mann–Whitney and Kruskal–

Wallis tests. Comparisons of qualitative variables were done by means of the χ^2 -test. All reported *P*-values represent two-tailed tests, with values of <0.05 which were considered statistically significant.

RESULTS

Patients and Controls

The study finally included 46 patients with NIUA, 26 women and 20 men (mean age, 40.5 ± 14.4 years) and 45 subjects who tolerated these drugs. The diagnosis was established by clinical history in five cases that had at least three episodes to three or more NSAIDs belonging to different chemical groups, and by DPT in 41 patients. The time between the drug intake and the appearance of the symptoms was <60 min in 29 out of the 46 cases (63.1%) and between 60 and 120 min in 17 cases (36.9%). The mean time between the NSAID reaction and obtaining the sample was 27.2 ± 34.8 months. Data are summarized in Table 1.

The drug inducing the reaction was ibuprofen in 21 cases (45.6%) and dipyron in 20 cases (43.5%), followed by diclofenac in 11 cases (10.8%), and ASA and paracetamol in 8 cases

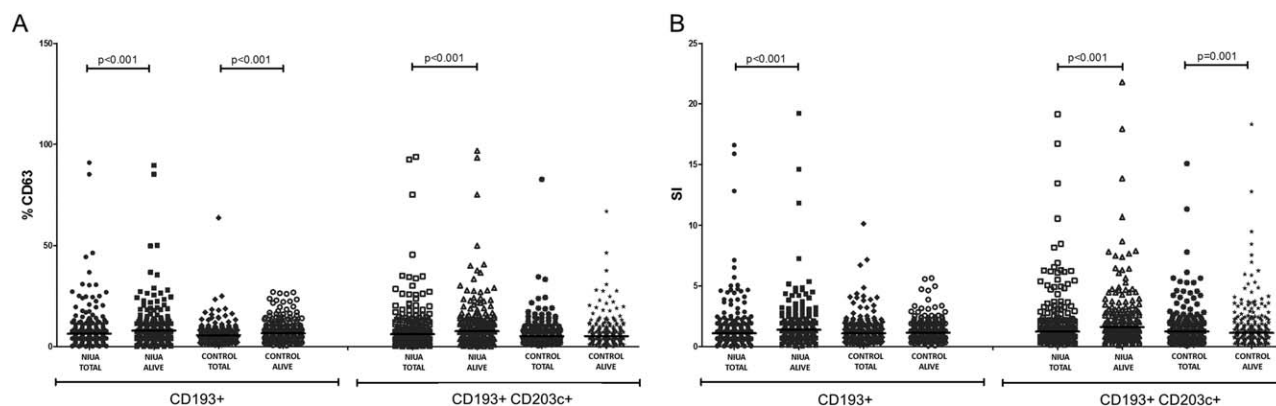


Figure 2. Influence of the elimination of dead cells on the analysis for both selection strategies in percentage of CD63 expressing cells (A) and SI value (B). Results are expressed for 20 individual determinations in 46 NIUA patients and 45 tolerant subjects. Positive and negative controls were eliminated from the analysis.

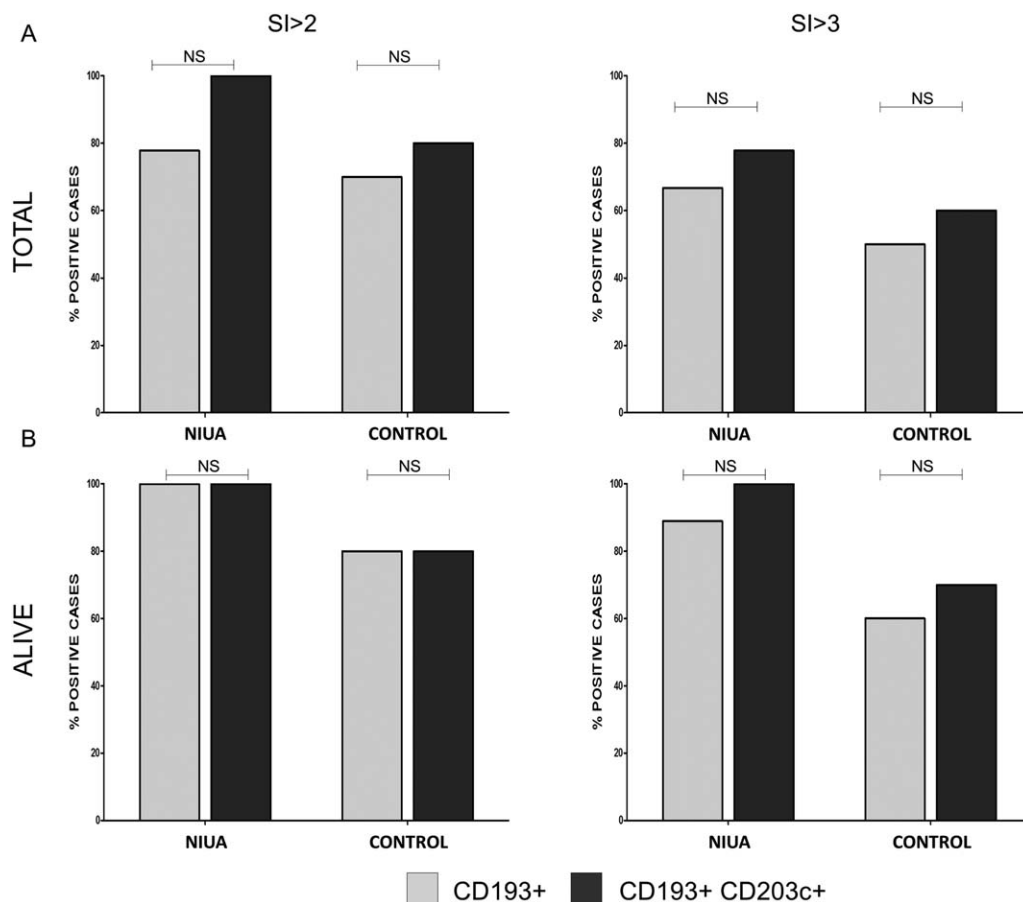


Figure 3. Percentage of positive cases in both strategies without (A) or with (B) exclusion of dead cells using a cut-off of 2 or 3 for the SI in 46 NIUA patients and 45 tolerant subjects. We considered a case as positive when the BAT SI was higher than the cut-off for at least one of the NSAIDs tested (NS, not significant differences using χ^2 -test).

(17.4%), with ketorolac in 3 cases (6.5%), naproxen in 2 cases (4.3%), and piroxicam in just 1 case (2.2%). Regarding the clinical manifestations, 12 cases (26.1%) had USS, 27 (58.7%) urticaria, 5 (10.8%) angioedema, and 2 (4.3%) UA. In the 12 cases with USS, the drugs involved were dipyrrone and/or ASA in five (41.6%), followed by diclofenac in three (25%). In the 34 cases having exclusively cutaneous symptoms, ibuprofen was the drug most frequently involved in 19 cases (55.9%).

In all the patients and controls, we first analyzed the percentage of nonactivation when samples were stimulated with anti-IgE (nonresponders); these were then eliminated for the subsequent analysis. We found a higher level of nonresponders in the group of patients (54.5%) compared to controls (9.1%) ($P < 0.001$).

We compared the BAT results using CD193⁺ or CD193⁺CD203c⁺ for basophil selection and found a significant increase in the percentage of basophils recovered using CD193⁺ in patients and controls ($P = 0.004$ and $P = 0.002$, respectively) (Fig. 1A), no differences in the percentage of CD63⁺-activated basophils (Fig. 1B), and significant increases in the SI when using CD193⁺CD203c⁺ in both patients and controls ($P = 0.004$ and $P = 0.017$, respectively) (Fig. 1C).

The influence of excluding or including dead cells in the BAT analysis using CD193⁺ or CD193⁺CD203c⁺ was also analyzed. In terms of percentage of activation, we found that the selection of living cells produced a significant increase in both patients and controls when using CD193⁺ cells ($P < 0.001$ in both cases) but only in patients when using CD193⁺CD203c⁺ cells ($P < 0.001$) (Fig. 2A). In terms of SI, there was an increase in patients for both CD193⁺ and CD193⁺CD203c⁺ ($P < 0.001$ for both), whereas in controls there was no change with CD193⁺ and a decrease with CD193⁺CD203c⁺ ($P = 0.001$) (Fig. 2B).

We then analyzed the influence of the two basophil characterization strategies and the presence of dead cells on BAT sensitivity at two different cut-off points (SI > 2 or SI > 3). We found that using CD193⁺CD203c⁺ increased the percentage of positive cases in patients and controls at SI > 2 (78.2 vs. 100% for patients and 70 vs. 80% for controls) and at SI > 3 (67.4 vs. 78.2% for NIUA and 51.1 vs. 60% for controls) when compared with CD193⁺ (Fig. 3A). When excluding dead cells, no changes were detected at SI > 2, whereas at SI > 3 there was an increase of 21.7% in patients and of 10% in controls (Fig. 3B). These results

Table 2. Sensitivity (percentage of positive cases in NIUA group) and specificity (percentage of negative cases in control group) of BAT with different NSAIDs considering positivity to at least one concentration for individual NSAID and to at least one NSAID for the total

		SENSITIVITY	SPECIFICITY	SENSITIVITY	SPECIFICITY
SI>2	DRUG	CD193 ⁺		CD193 ⁺ ALIVE	
	ASA	43.5%	60%	43.5%	51.1%
	DICLOFENAC	21.7%	91.1%	21.7%	80%
	NAPROXEN	45.6%	51.1%	45.6%	31.1%
	PARACETAMOL	34.8%	60%	55.5%	20%
	DIPYRONE	32.6%	68.8%	78.2%	60%
	IBUPROFEN	55.5%	48.8%	67.4%	48.8%
	TOTAL	78.2%	31.1%	100%	20%
		CD193 ⁺ CD203c ⁺		CD193 ⁺ CD203c ⁺ ALIVE	
	ASA	89.1%	20%	78.2%	40%
	DICLOFENAC	32.6%	68.8%	55.5%	60%
	NAPROXEN	67.4%	31.1%	76.1%	42.2%
	PARACETAMOL	43.5%	20%	43.5%	31.1%
	DIPYRONE	45.6%	68.8%	55.5%	80%
	IBUPROFEN	67.4%	68.8%	89.1%	68.8%
	TOTAL	100%	20%	100%	20%
SI>3	DRUG	CD193 ⁺		CD193 ⁺ ALIVE	
	ASA	21.7%	91.1%	10.8%	91.1%
	DICLOFENAC	10.8%	91.1%	21.7%	100%
	NAPROXEN	10.8%	80%	10.8%	100%
	PARACETAMOL	21.7%	60%	21.7%	60%
	DIPYRONE	32.6%	91.1%	55.5%	91.1%
	IBUPROFEN	45.6%	80%	55.5%	60%
	TOTAL	67.4%	51.1%	89.1%	40%
		CD193 ⁺ CD203c ⁺		CD193 ⁺ CD203c ⁺ ALIVE	
	ASA	43.5%	68.8%	32.6%	60%
	DICLOFENAC	21.7%	80%	21.7%	68.8%
	NAPROXEN	45.6%	60%	43.5%	80%
	PARACETAMOL	21.7%	68.8%	45.6%	51.1%
	DIPYRONE	32.6%	80%	43.5%	68.8%
	IBUPROFEN	55.5%	60%	89.1%	51.1%
	TOTAL	78.2%	40%	100%	31.1%

Results were presented using a SI cut-off of 2 or 3.

indicate that using CD193⁺CD203⁺, excluding dead cells, is the best approach.

The results of the BAT sensitivity (percentage of positive cases into the NIUA patients) and specificity (percentage of negative cases into the control group) for each NSAID (Table 2) showed that the best results were found with dipyrone when dead cells were excluded and the worst with paracetamol followed by diclofenac. Considering the results for total sensitivity and specificity, the sensitivity could reach 100%, whereas the specificity fell to 20%.

DISCUSSION

Over recent years, NSAIDs have become the drugs most frequently involved in hypersensitivity reactions, with NIUA being the most common diagnosis (2,31). The evaluation of possibly affected patients is difficult, and diagnosis is based on the clinical history, though in many cases DPT is also neces-

sary (14,15), with the BAT being used for diagnoses but with variable results (24). In this study, we evaluated an important number of well-characterized patients with NIUA and controls, taking into account different technical approaches and using a wide panel of NSAIDs. Even considering all these variables, we were unable to obtain an optimal sensitivity/specificity ratio that could recommend this method for diagnosing this group of patients. This is probably related with the mechanism underlying NIUA where IgE is not implicated.

In this study, we found a significantly higher number of nonresponders in the patients (54.5%) compared to the controls (9.1%) and compared to the previous reports where no more than 20% of all donors completely fail to release histamine in response to anti-IgE (42). Although a translational or post-translational alteration in the expression of the tyrosine kinases lyn and syk has been described in nonreleasing human basophils (43), the reason for the increase in NSAID hypersensitivity patients is unknown and needs further investigations.

The different approaches for performing BAT may influence the results, i.e. the type of sample, such as whole blood, buffy coat, or total leukocytes. In this study, we used whole blood which, although easily manageable, could have the inconvenience of giving false-positive results owing to the CD63 expression in dendritic cells and platelets (28). However, we avoided this by using two specific markers for basophil selection as CD193, highly expressed in basophils and eosinophils but also in Th2 and Treg lymphocytes (44) and CD203c expressed only in basophils (37,38). The CD203c increased its expression after basophil activation and for this reason it has been widely used as basophil activation marker; however, its usefulness is a matter of debate owing to its constitutive expression (40). As it is not known whether the two markers are equivalent, we compared CD193⁺ with CD193⁺CD203c⁺ combination for selecting basophils and found that although there was a decrease of the percentage of basophils recovered when using the combination, technically the selection of the cells was easier and more specific than using CD193 alone. This can be explained because CD193 can be present in other cell subtypes (44) and is downregulated with basophil activation (45), unlike CD203c which is upregulated (40). Moreover, the basophils selection strategy used also influences the basophil activation results, as an increase in the SI using the CD193⁺CD203c⁺ strategy was found. Although no data have yet been published, we considered it important to analyze the influence of the presence of dead cells on the BAT results. We found that exclusion of dead cells increases the percentage of basophil activation as measured by the percentage of cells expressing CD63 and by the SI. These results indicate that a better sensitivity and specificity were obtained with CD193⁺CD203c⁺ and dead cell exclusion. This is important, and not only for NSAID hypersensitivity, and it needs to be explored in other drug hypersensitivity reactions.

However, taking into account all these technical considerations and considering BAT positivity to all NSAIDs (taking as positive case when BAT was positive at least with one NSAID), we found that although 100% sensitivity could be obtained in the NIUA group, the specificity dramatically decreased to 31.1%, in the control group, when using both markers and excluding dead cells. These results, although better than those obtained by others concerning sensitivity (42.8 or 54.8%), showed important differences regarding specificity (100 or 88.9%) (27,30). However, in the particular case of ASA, our results are in agreement with another study that detected 30% ASA-induced CD63 expression in patients with aspirin-exacerbated respiratory disease (29). Nevertheless, all these data indicate that the main pitfall of BAT to evaluate NSAIDs hypersensitivity is the false-positive results, reflected in the low specificity obtained, which may be explained by their capacity to interact pharmacologically with basophils, inducing their activation in both NSAID-intolerant and NSAID-tolerant subjects (46).

Analyzing the BAT results for each NSAID, we consider it important to mention the lower sensitivity obtained with diclofenac (21.7%), a result that differs from other studies

detecting a sensitivity of 43.3% (28). This low sensitivity could be related to the capacity of diclofenac to induce basophil degranulation without CD63 expression (47). The fact whether this phenomenon happens with other NSAIDs is at present unknown.

CONCLUSIONS

Summarizing, BAT does not appear useful for the evaluation of patients with NIUA and although the sensitivity can be high the low specificity makes this method unsound. However, the results of this study show promising methodological possibilities, such as the use of the combination of CD193 and CD203c as basophil selection markers and the exclusion of dead cells from the cytometry analysis. These incorporations may well improve the BAT for the evaluation of IgE hypersensitivity reactions to drugs.

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LITERATURE CITED

1. Faich GA. Adverse-drug-reaction monitoring. *N Engl J Med* 1986;314:1589–1592.
2. Dona I, Blanca-Lopez N, Cornejo-García JA, Torres MJ, Laguna JJ, Fernandez J, Rosado A, Rondon C, Campo P, Agundez JA, et al. Characteristics of subjects experiencing hypersensitivity to non-steroidal anti-inflammatory drugs: Patterns of response. *Clin Exp Allergy* 2011;41:86–95.
3. Kowalski ML, Makowska JS, Blanca M, Baybek S, Bochenek G, Bousquet J, Bousquet P, Celik G, Demoly P, Gomes ER, et al. Hypersensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs) - classification, diagnosis and management: review of the EAACI/ENDA(®) and GA2LEN/HANNA*. *Allergy* 2011;66:818–829.
4. Quirarte J, Blanco C, Castillo R, Delgado J, Carrillo T. Intolerance to nonsteroidal antiinflammatory drugs: Results of controlled drug challenges in 98 patients. *J Allergy Clin Immunol* 1996;98:678–685.
5. Carmona MJ, Blanca M, García A, Fernandez S, Burgos F, Miranda A, Vega JM, García J. Intolerance to piroxicam in patients with adverse reactions to nonsteroidal antiinflammatory drugs. *J Allergy Clin Immunol* 1992;90:873–879.
6. de Paramo BJ, Gancedo SQ, Cuevas M, Camo IP, Martín JA, Cosmes EL. Paracetamol (acetaminophen) hypersensitivity. *Ann Allergy Asthma Immunol* 2000;85:508–511.
7. Blanca M, Perez E, García JJ, Miranda A, Terrados S, Vega JM, Suar R. Angioedema and IgE antibodies to aspirin: A case report. *Ann Allergy* 1989;62:295–298.
8. Harrer A. Diclofenac-specific monoclonal antibodies show variable cross-reactivity to diclofenac metabolites. *EAACI Abstract Book* 2007:35–36.
9. Scala E, Giani M, Pirrotta L, Guerra EC, Locanto M, De Pita O, Puddu P. Selective severe anaphylactic reaction due to ketorolac tromethamine without nonsteroidal anti-inflammatory drug intolerance. *J Allergy Clin Immunol* 2001;107:557.
10. Himly M, Jahn-Schmid B, Pitterschatzsch K, Bohle B, Grubmayr K, Ferreira F, Ebner H, Ebner C. IgE-mediated immediate-type hypersensitivity to the pyrazolone drug propyphenazone. *J Allergy Clin Immunol* 2003;111:882–888.
11. Kowalski ML, Bienkiewicz B, Woszczek G, Iwaszkiewicz J, Poniatowska M. Diagnosis of pyrazolone drug sensitivity: Clinical history versus skin testing and in vitro testing. *Allergy Asthma Proc* 1999;20:347–352.
12. Fontaine C, Bousquet PJ, Demoly P. Anaphylactic shock caused by a selective allergy to celecoxib, with no allergy to rofecoxib or sulfamethoxazole. *J Allergy Clin Immunol* 2005;115:633–634.
13. Szczeklik A, Gryglewski RJ, Czerniawska-Mysik G. Relationship of inhibition of prostaglandin biosynthesis by analgesics to asthma attacks in aspirin-sensitive patients. *Br Med J* 1975;1:67–69.
14. Szczeklik A, Stevenson DD. Aspirin-induced asthma: Advances in pathogenesis, diagnosis, and management. *J Allergy Clin Immunol* 2003;111:913–921; quiz 922.
15. Stevenson DD, Simon RA. Sensitivity to aspirin and nonsteroidal anti-inflammatory drugs. In: Middleton E, editor. *Allergy: Principles & Practice*. St. Louis: Mosby; 1998.
16. Messaad D, Sahla H, Benahmed S, Godard P, Bousquet J, Demoly P. Drug provocation tests in patients with a history suggesting an immediate drug hypersensitivity reaction. *Ann Intern Med* 2004;140:1001–1006.

17. Nizankowska-Mogilnicka E, Bochenek G, Mastalerz L, Swierczynska M, Picado C, Scadding G, Kowalski ML, Setkowicz M, Ring J, Brockow K, et al. EAACI/GA2LEN guideline: Aspirin provocation tests for diagnosis of aspirin hypersensitivity. *Allergy* 2007;62:1111–1118.
18. Sanz ML, Gamboa PM, Antepara I, Uasuf C, Vila L, Garcia-Aviles C, Chazot M, De Weck AL. Flow cytometric basophil activation test by detection of CD63 expression in patients with immediate-type reactions to betalactam antibiotics. *Clin Exp Allergy* 2002;32:277–286.
19. Torres MJ, Padial A, Mayorga C, Fernandez T, Sanchez-Sabate E, Cornejo-Garcia JA, Antunez C, Blanca M. The diagnostic interpretation of basophil activation test in immediate allergic reactions to betalactams. *Clin Exp Allergy* 2004;34:1768–1775.
20. Aranda A, Mayorga C, Ariza A, Dona I, Blanca-Lopez N, Canto G, Blanca M, Torres MJ. IgE-mediated hypersensitivity reactions to methylprednisolone. *Allergy* 2010;65:1376–1380.
21. Aranda A, Mayorga C, Ariza A, Dona I, Rosado A, Blanca-Lopez N, Andreu I, Torres MJ. In vitro evaluation of IgE-mediated hypersensitivity reactions to quinolones. *Allergy* 2011;66:247–254.
22. Abuaf N, Rajoely B, Ghazouani E, Levy DA, Pecquet C, Chabane H, Leynadier F. Validation of a flow cytometric assay detecting in vitro basophil activation for the diagnosis of muscle relaxant allergy. *J Allergy Clin Immunol* 1999;104:411–418.
23. Ebo DG, Bridts CH, Hagendorens MM, Mertens CH, De Clerck LS, Stevens WJ. Flow-assisted diagnostic management of anaphylaxis from rocuronium bromide. *Allergy* 2006;61:935–939.
24. Gamboa PM, Sanz ML, Caballero MR, Antepara I, Urrutia I, Jauregui I, Gonzalez G, Dieguez I, De Weck AL. Use of CD63 expression as a marker of in vitro basophil activation and leukotriene determination in metamizol allergic patients. *Allergy* 2003;58:312–317.
25. Gomez E, Blanca-Lopez N, Torres MJ, Requena G, Rondon C, Canto G, Blanca M, Mayorga C. Immunoglobulin E-mediated immediate allergic reactions to dipyrone: Value of basophil activation test in the identification of patients. *Clin Exp Allergy* 2009;39:1217–1224.
26. Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. In vitro allergy diagnosis: Should we follow the flow? *Clin Exp Allergy* 2004;34:332–339.
27. Rodriguez-Trabado A, Camara-Hijon C, Ramos-Cantarino A, Porcel-Carreno SL, Jimenez-Timon S, Pereira-Navarro G, Hernandez-Arbeiza FJ, Fernandez-Pereira L. Basophil activation test for the in vitro diagnosis of nonsteroidal anti-inflammatory drug hypersensitivity. *Allergy Asthma Proc* 2008;29:241–249.
28. Gamboa P, Sanz ML, Caballero MR, Urrutia I, Antepara I, Esparza R, de Weck AL. The flow-cytometric determination of basophil activation induced by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is useful for in vitro diagnosis of the NSAID hypersensitivity syndrome. *Clin Exp Allergy* 2004;34:1448–1457.
29. Celik GE, Schroeder JT, Hamilton RG, Saini SS, Adkinson NF. Effect of in vitro aspirin stimulation on basophils in patients with aspirin-exacerbated respiratory disease. *Clin Exp Allergy* 2009;39:1522–1531.
30. De Weck AL, Sanz ML, Gamboa PM, Aberer W, Blanca M, Correia S, Erdman S, Jeremann JM, Kanny G, Kowalski M, et al. Nonsteroidal anti-inflammatory drug hypersensitivity syndrome. A multicenter study. I. Clinical findings and in vitro diagnosis. *J Investig Allergol Clin Immunol* 2009;19:355–369.
31. Szczeklik A N-ME, Sanak M. Hypersensitivity to aspirin and nonsteroidal anti-inflammatory drugs. In: Adkinson NF, Bochner BS, Holgate ST, Lemanske RF, Simons, FE, editors. *Middleton's Allergy Principles and Practice*. Philadelphia: Mosby; 2009. pp 1227–1243.
32. Blanca-Lopez N, Andreu I, Torres Jaen MJ. Hypersensitivity reactions to quinolones. *Curr Opin Allergy Clin Immunol* 2011;11:285–291.
33. MacGlashan D, Jr, Schroeder JT. Functional consequences of FcεpsilonRIα up-regulation by IgE in human basophils. *J Leukoc Biol* 2000;68:479–486.
34. MacGlashan D, Jr, Xia HZ, Schwartz LB, Gong J. IgE-regulated loss, not IgE-regulated synthesis, controls expression of FcεpsilonRI in human basophils. *J Leukoc Biol* 2001;70:207–218.
35. Sainte-Laudy J, Boumediene A, Touraine F, Orsel I, Brianchon C, Bonnaud F, Cogne M. Use of both CD63 up regulation and IgE down regulation for the flow cytometric analysis of allergen induced basophil activation. Definition of an activation index. *Inflamm Res* 2007;56:291–296.
36. Abuaf N, Rostane H, Rajoely B, Gaouar H, Autegarden JE, Leynadier F, Girot R. Comparison of two basophil activation markers CD63 and CD203c in the diagnosis of amoxicillin allergy. *Clin Exp Allergy* 2008;38:921–928.
37. Hausmann OV, Gentinetta T, Fux M, Ducrest S, Pichler WJ, Dahinden CA. Robust expression of CCR3 as a single basophil selection marker in flow cytometry. *Allergy* 2011;66:85–91.
38. de Weck AL, Sanz ML, Gamboa PM, Aberer W, Bienvenu J, Blanca M, Demoly P, Ebo DG, Mayorga L, Monneret G, et al. Diagnostic tests based on human basophils: More potentials and perspectives than pitfalls. *Int Arch Allergy Immunol* 2008;146:177–189.
39. Sturm EM, Kranzelbinder B, Heinemann A, Groselj-Strele A, Aberer W, Sturm GJ. CD203c-based basophil activation test in allergy diagnosis: Characteristics and differences to CD63 upregulation. *Cytometry B Clin Cytom* 2010;78B:308–318.
40. MacGlashan DW, Jr. Basophil activation testing. *J Allergy Clin Immunol* 2013;132:777–787.
41. Blanca-Lopez N, J Torres M, Dona I, Campo P, Rondon C, Seoane Reula ME, Salas M, Canto G, Blanca M. Value of the clinical history in the diagnosis of urticaria/angioedema induced by NSAIDs with cross-intolerance. *Clin Exp Allergy* 2013;43:85–91.
42. Nguyen KL, Gillis S, MacGlashan DW, Jr. A comparative study of releasing and non-releasing human basophils: Nonreleasing basophils lack an early component of the signal transduction pathway that follows IgE cross-linking. *J Allergy Clin Immunol* 1990;85:1020–1029.
43. Lavens-Phillips SE, MacGlashan DW, Jr. The tyrosine kinases p53/56lyn and p72syk are differentially expressed at the protein level but not at the messenger RNA level in nonreleasing human basophils. *Am J Respir Cell Mol Biol* 2000;23:566–571.
44. Monneret G. CCR3 for basophil activation test: A necessary but insufficient step. *Clin Exp Allergy* 2010;40:953; author reply 954.
45. Sainte-Laudy J, Ouk C. Use of lipid rafting for the analysis of human basophil activation by flow cytometry. *Inflamm Res* 2010;59:S193–S195.
46. Rodriguez Trabado A, Camara Hijon C, Porcel Carreno SL, Rodriguez Martin E, Fletes Peral C, Pereira Navarro G, Jimenez Timon S, Hernandez Arbeiza FJ, Fernandez Pereira L. Anaphylaxis caused by cloxacillin: Diagnosis with seriated analysis by way of basophil activation test. *Allergy Asthma Proc* 2006;27:269–272.
47. Malbran A, Yeyati E, Rey GL, Galassi N. Diclofenac induces basophil degranulation without increasing CD63 expression in sensitive patients. *Clin Exp Immunol* 2007;147:99–105.

Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	To evaluate the potential value of the basophil activation test (BAT) using different basophil markers in the diagnosis of patients with cross-intolerance to NSAIDs and cutaneous symptoms.
1.2. Keywords	Hypersensitivity, non-steroidal anti-inflammatory drugs, idiosyncratic reactions, basophils, CD63, CCR3, CD203c.
1.3. Experiment variables	Basophil activation
1.4. Organization name and address	Research Laboratory, Carlos Haya Hospital-IBIMA Pl. Hospital Civil, 29009 Malaga, Spain
1.5. Primary contact name and email address	Cristobalina Mayorga mayorga.lina@gmail.com
1.6. Date or time period of experiment	Three-year period (2010-2013)
1.7. Conclusions	The results indicate that using CCR3 ⁺ CD203 ⁺ excluding dead cells is the best approach for BAT; although the BAT is not recommended for the diagnosis of patients with cross-intolerance to NSAIDs due to its low sensitivity and specificity.
1.8. Quality control measures	It have been used: - Positive and negative controls to determine CD63 expression. - A group of control subjects. - Isotopic controls to determine the gate boundaries.
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	Patients with multiple NSAID-induced hypersensitivity including the following symptoms: urticaria, angioedema, urticaria and angioedema (UA), or urticaria and systemic symptoms (USS).
2.1.1.2. Biological sample source description	Heparinized whole blood
2.1.1.3. Biological sample source organism description	Human
2.1.2.2. Environmental sample location	Málaga, Spain.
2.3. Sample treatment description	One hundred µL of heparinized whole blood was aliquoted per test and 20 µL of stimulation buffer was added and incubated for 10 min at 37°C in a water bath. After this, 100 µL of the washing solution was added to the negative control tube, and 100 µL of fMLP (chemotactic peptide N-Formyl-Met- Leu-Phe) or anti IgE (BD Pharmingen, New Jersey, USA) to the positive control tube. One hundred µL of different NSAIDs was added to the different samples. ASA at 1, 0.5, 0.2 and 0.1 mg/mL, diclofenac at 0.3, 0.15, 0.06 and 0.03 mg/mL, naproxen at 1, 0.5 0.2 and 0.1 mg/mL, dipyrrone at 5, 2.5, 0.25 and 0.1 mg/mL, and ibuprofen at 5, 1.5, 0.75 and 0.15 mg/mL (all from Sigma Aldrich, Saint Louis, MO) and paracetamol (Novartis Farmaceutica SA, Barcelona, Spain) at 5, 1, 0.5 and 0.1 mg/mL. The samples were incubated for 30 min at 37 °C in a water bath. The degranulation was stopped by incubating the samples on ice for 5 min and then 3 µL of

	monoclonal antibodies were added to each tube. After this, 2mL of pre-warmed lysing solution was added and incubated for 10 min at room temperature. After centrifuging and washing, the cells were analyzed.																												
2.4. Fluorescence reagent(s) description	anti-CD63-FITC, CD203c-PE, CCR3-APC (Caltag Laboratories, Burlingame, CA) and Live/Dead Fixable FarRed Dead Cell Stain Kit (Invitrogen, Ltd. Paisley, UK)																												
3.1. Instrument manufacturer	Becton-Dickinson Bioscience, San Jose, CA																												
3.2. Instrument model	FACSCalibur																												
3.3. Instrument configuration and settings	One 488nm laser. <table><thead><tr><th></th><th>Voltage</th><th>AmpGain</th><th>Mode</th></tr></thead><tbody><tr><td>FSC</td><td>E00</td><td>4.04</td><td>Lin</td></tr><tr><td>SSC</td><td>392</td><td>1.57</td><td>Lin</td></tr><tr><td>FL1</td><td>777</td><td>1.00</td><td>Log</td></tr><tr><td>FL2</td><td>551</td><td>1.00</td><td>Log</td></tr><tr><td>FL3</td><td>798</td><td>1.00</td><td>Log</td></tr><tr><td>FL4</td><td>770</td><td>1.00</td><td>Log</td></tr></tbody></table>		Voltage	AmpGain	Mode	FSC	E00	4.04	Lin	SSC	392	1.57	Lin	FL1	777	1.00	Log	FL2	551	1.00	Log	FL3	798	1.00	Log	FL4	770	1.00	Log
	Voltage	AmpGain	Mode																										
FSC	E00	4.04	Lin																										
SSC	392	1.57	Lin																										
FL1	777	1.00	Log																										
FL2	551	1.00	Log																										
FL3	798	1.00	Log																										
FL4	770	1.00	Log																										
4.1. List-mode data files	*Deposited in http://flowrepository.org The repository identifier: http://flowrepository.org/id/FR-FCM-ZZ93.																												
4.2. Compensation description	Compensations have been done for the 4 colors (FITC, PE, PerCP and APC) in each experiment. FL1 - 0.0% FL2 FL2 – 30.0% FL1 FL2 – 1.4% FL3 FL3 – 11.3% FL2 FL3 – 10.8% FL4 FL4 – 0.0% FL3																												
4.3. Data transformation details	IS = % CD63 in samples / % CD63 in negative control.																												
4.4.1. Gate description	The diagram illustrates the gate selection process in four stages: Initial Population: A scatter plot of SSC-H vs FSC-H showing a population of Lymphocytes (LNF) at 95.7%. Live Cell Selection: A histogram of FL3-H (LIVE-DEAD FarRed) showing the selection of ALIVE cells (98.8%) and exclusion of DEAD cells (1.14%). CCR3+ Selection: A scatter plot of FL4-H (CCR3 APC) vs SSC-H showing the selection of CCR3+ cells at 3.41%. CCR3+CD203c+ Selection: A scatter plot of FL4-H (CCR3 APC) vs FL3-H (CD203c PE) showing the final selection of CCR3+CD203c+ cells at 3.17% (top right) and 2.89% (bottom right).																												
4.4.2. Gate statistics																													

4.4.3. Gate boundaries	
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Notes

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