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To cite this article: Jamma Li, Oliver Giles Best, Stephen P. Mulligan & Suran Loshana Fernando (2016): Serum from a subset of patients with chronic lymphocytic leukemia and large local reactions to mosquito bites induces upregulation of CD63 surface expression on basophils in atopic donors, *Leukemia & Lymphoma*, DOI: [10.3109/10428194.2016.1173208](https://doi.org/10.3109/10428194.2016.1173208)

To link to this article: <http://dx.doi.org/10.3109/10428194.2016.1173208>

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LETTER TO THE EDITOR

Serum from a subset of patients with chronic lymphocytic leukemia and large local reactions to mosquito bites induces upregulation of CD63 surface expression on basophils in atopic donors

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ARTICLE HISTORY Received 22 December 2015; revised 20 March 2016; accepted 26 March 2016

Reactions to mosquito bites are immunological reactions that are due to sensitization to mosquito salivary proteins.[1] Large local reactions (LLRs) are areas of erythema and swelling from 2 to over 10 cm, which develop within a few hours of the bite, progress over 8–12 h, and resolve in 3–10 d [Figure 1(A)].[2] LLRs are painful and may cause significant distress. They seldom occur in adults, however, certain adult populations have increased prevalence of LLRs including those with chronic lymphocytic leukemia (CLL).[3] Data suggest that prevalence in CLL varies between 2 and 45%.[4]

LLRs are thought to be primarily mediated through the high-affinity IgE receptor on mast cells and basophils (FcεRI),[1] as distinct from the FcεRII (CD23).[5] Other contributing mechanisms have been proposed, including Arthus-type reactions,[6] and T-cell mediated hypersensitivity.[7] CLL is a heterogeneous disorder associated with autoantibody formation and autoimmune disease in approximately 25% of patients.[8] We hypothesized that the sera of CLL patients, who experience LLRs to mosquito bites, contain antibodies that interact with the FcεRI and activate mast cells and basophils. We used basophil activation testing (BAT) to demonstrate interaction of CLL serum with the FcεRI. Patients with CLL and LLRs to mosquito bites within the last 12 months were recruited from the weekly CLL clinic over four weeks at Royal North Shore Hospital, Sydney, Australia. A diagnosis of CLL was established by IWCLL criteria [9] from clinical history, a peripheral B lymphocyte count exceeding $5 \times 10^9/L$, and clonality demonstrated on flow cytometry with a typical CLL phenotype.

A diagnosis of a LLR was accepted based on clinical history of a reaction within the last 12 months following a mosquito bite characterized by large swelling and redness around the bite mark at least 2 cm wide, which develops within a few hours of the bite, progresses over 8–12 h,

and resolves within 3–10 d. Patients with systemic reactions, concurrent autoimmune disease or atopy were excluded from the study.

Five patients with CLL and a clinical history of LLRs were identified and included in the study (study population). Informed consent was obtained. All sera were collected within 12 months of the experiments.

Two basophil donors were included in the study. One donor was atopic with a clinical history of asthma and allergic rhinitis, but no other significant past medical history. The second donor was non-atopic, and had no significant past medical history. Informed consent was obtained.

Whole blood from both basophil donors was collected in lithium heparin. CD63 surface expression was measured within 3 h of collection using premixed monoclonal antibodies CD63 PE, CD123 FITC and HLA-DR PerCP (Becton Dickinson, Franklin Lakes, NJ). Whole blood from each basophil donor (100 µL) was incubated at 37 °C with 20 µL of basophil stimulation buffer (BSB) containing 4 ng/mL IL-3 (Sigma-Aldrich, St Louis, MO) in RPMI-1640 medium (Life Technologies, Carlsbad, CA), and 100 µL aliquots of serum collected in lithium heparin from each patient in the study population for 20 min. As a negative control, donor basophils were incubated with 20 µL of BSB and 100 µL of RPMI-1640 medium. As a positive control, donor basophils were incubated with 20 µL of BSB and 100 µL of 1 µM N-formyl-Met-Leu-Phe chemotactic peptide (fMLP) (Sigma-Aldrich, St Louis, MO).

Reactions were stopped by adding 20 µL of 20 mM EDTA (Sigma-Aldrich, St Louis, MO). Cells were stained with 20 µL of premixed monoclonal antibodies CD63 PE, CD123 FITC and HLA-DR PerCP at room temperature in the dark for 15 min. Red cells were lysed by adding 2 mL of FACS lysing solution (Becton Dickinson, Franklin Lakes, NJ) and then centrifuged. Cells were

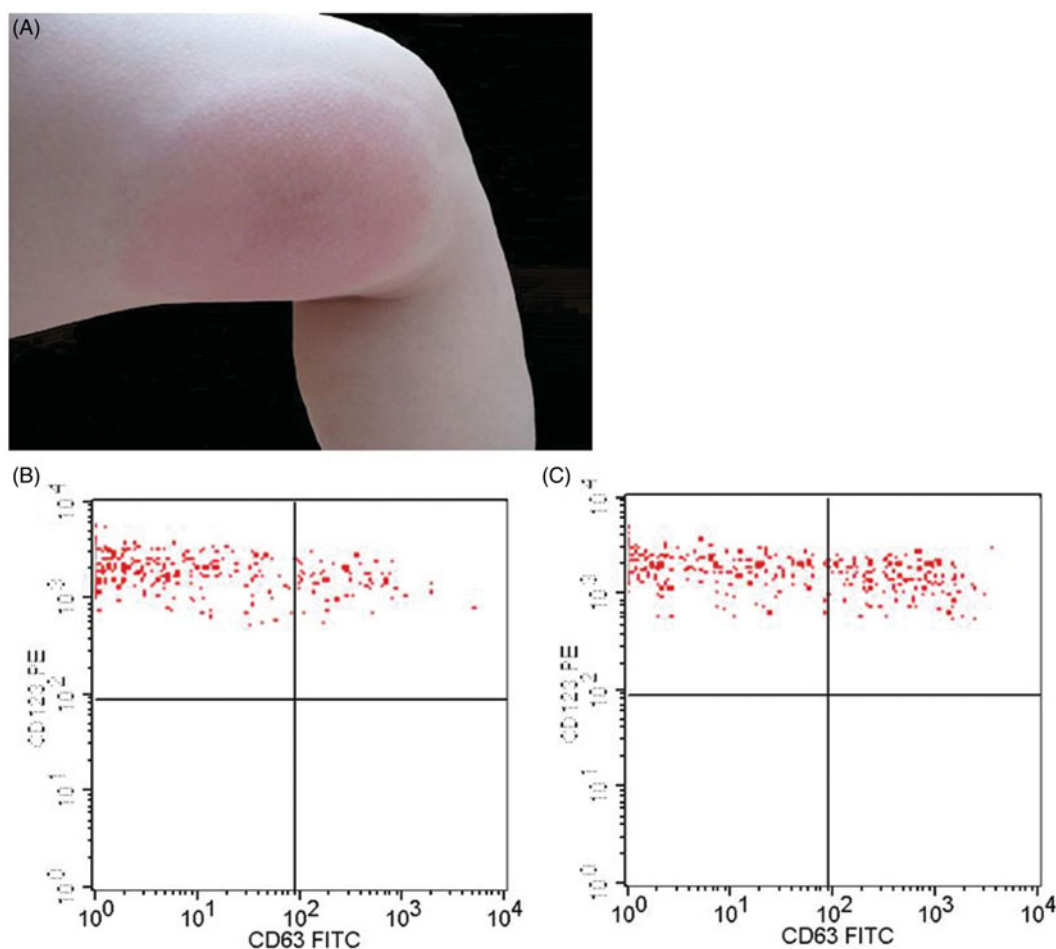


Figure 1. (A) Large local reaction to a mosquito bite. (B) Significant upregulation of CD63 by serum from CLL Patient 1. (C) Significant upregulation of CD63 by serum from CLL Patient 4.

washed with 2 mL PBS and fixed in 300 μ L 0.5% paraformaldehyde.

Flow analysis was performed using a Becton Dickinson FACSCalibur flow cytometer and Cell Quest Pro software (Becton Dickinson, Franklin Lakes, NJ). Basophils were identified as a single population of cells that stained positive for CD123 but not HLA-DR. Upregulation of CD63 expression was measured by the percentage of basophils positive for CD63 above the background positivity in the BSB control (negative control). All BATs were repeated three times on six separate days with serum from each patient in the study population.

The number of serum samples in our study population precluded definition of a cutoff value. Therefore, we applied cutoff values of 5.1 and 7.7% determined in previous studies of chronic urticaria [10,11] and performed statistical analyses comparing the levels of basophil activation between atopic and non-atopic donor basophils for each of the serum samples in the study population. Statistical analyses were conducted using a Student's *t*-test with values of $p < 0.05$ considered significant.

Patient characteristics are listed in Table 1. Basophils from the atopic donor showed significantly higher

($p = 0.002$) CD63 upregulation after stimulation with fMLP ($27.17\% \pm 2.71$), than basophils from the non-atopic donor ($5.3\% \pm 1.33$). No activation of basophils from either donor was observed in the negative control.

Using basophils from the atopic donor, sera from two of five patients in the study population [Figures 1(B) and 1(C)] induced CD63 upregulation above either the 5.1 or 7.7% cutoff, while levels of activation in the remaining three samples were below 5.1% [Table 1]. Serum from CLL Patient 1 induced activation of $9.66\% \pm 1.55$ ($p = 0.001$). Serum from CLL Patient 4 induced activation of $36.8\% \pm 8.39$ ($p = 0.006$). No sera from the study population induced activation above the 5.1% cutoff in basophils from the non-atopic donor. We also observed a significantly higher ($p < 0.01$) percentage of basophils activated by these two sera in the atopic donor compared with the non-atopic donor sample.

Our finding that sera from the study population induces CD63 upregulation on basophils from an atopic donor, and not on those from a non-atopic donor is consistent with current literature.[10,11] Basophils from atopic donors have a higher surface density of Fc ϵ RI.

Sera from two patients activated basophils. This is novel indirect evidence that sera from these two patients

Table 1. Study population: patient characteristics.

CLL patient	Age	Sex (M/F)	% Activation		Time from diagnosis of CLL	Treatment history	Time from last treatment to serum collection	Lymphocyte count ($\times 10^9/L$)	Eosinophil count ($\times 10^9/L$)	IgG (g/L)	B2-micro-globulin (mg/L)	CLL clone present	EBV serology	IgE (kU/L)	sIgE (mosquito) (kUA/L)	sIgE (food mix) (kUA/L)	sIgE (dust mite) (kUA/L)	sIgE (mould mix) (kUA/L)	sIgE (grass mix) (kUA/L)	sIgE (animal mix) (kUA/L)	Tryptase ($\mu g/L$)
			of non-atopic basophils	of atopic basophils																	
1	75	F	0.47 $\pm$ 0.46	9.66 $\pm$ 1.15 ($p=0.001$)	10 years	FC	3 years	239.3	0.1	7.7	3.9	Yes	IgG positive	34	<0.01	<0.35	<0.35	<0.35	<0.35	<0.35	7.3
2	79	F	0	0.59 $\pm$ 0.51 ($p=0.19$)	15 years	FCR	3.5 years	0.9	0.0	10.85	2.5	Yes	IgG positive	<2	<0.01	<0.35	<0.35	<0.35	<0.35	<0.35	2.3
3	51	M	0	0	12 years	FCR Ibrutinib	Currently on treatment	155.8	0.1	11.66	2.3	Yes	IgG positive	<2	<0.01	<0.35	<0.35	<0.35	<0.35	<0.35	3.9
4	71	M	0.7 $\pm$ 0.41	36.8 $\pm$ 8.39 ($p=0.006$)	11 years	FCR	Currently on treatment	1.6	0.0	10.05	2.1	Yes	IgG positive	<2	<0.01	<0.35	<0.35	<0.35	<0.35	<0.35	5.8
5	41	M	0.26 $\pm$ 0.26	0.84 $\pm$ 0.73 ($p=0.27$)	7 years	R DAC EDOCH	Currently on treatment	0.9	0.0	7.12	2.8	Yes	IgG positive	461	0.03	<0.35	<0.35	<0.35	<0.35	<0.35	4.1

FC: fludarabine + cyclophosphamide; R: rituximab; DAC: dexamethasone + Ara C + cisplatin; EDOCH: etoposide, dexamethasone, vincristine, cyclophosphamide, doxorubicin.

contain factors that lead to basophil activation. We speculate that the factors in the sera which lead to basophil activation are autoantibodies, and hence a potentially novel autoimmune mechanism in CLL. We excluded the presence of confounding antibodies. Specific IgE testing to mosquito and common inhalant and food allergens in addition to total IgE exclude these as triggers for basophil activation [Table 1]. Our study excluded patients with concurrent autoimmune disease, as IgG autoantibodies to FcεRI have been identified in several cases of autoimmune blistering diseases, dermatomyositis and systemic lupus erythematosus.[12] Our study population did not have positive direct antiglobulin tests, or thyroid autoantibodies, although neither is known to bind the FcεRI. Serum eosinophil counts and tryptase levels were all within normal limits.

No upregulation of CD63-positive basophils from the atopic donor was observed for three out of five sera. There are many possible explanations for this. Presence of antibodies to the FcεRI may only predict the severity of LLRs, paralleling chronic urticaria, where the presence of IgE antibodies or antibodies to the FcεRI predicts more severe disease.[13–15] We used a prescribed questionnaire to obtain retrospective clinical history from our study patients, and did not ask specific details in relation to their LLRs. The structure of our questionnaire meant that we were not able to determine whether the two patients with sera that significantly induced basophil activation had more severe LLRs in terms of size and duration compared to the remainder of the study population.

Further, delayed processing time may have contributed to breakdown of antibodies. Also, there may be upregulation of other markers of basophil activation such as CD203c or CD300a, and not CD63, and we would be interested to determine whether there is any significant difference using CD203c or CD300a in a future study. Other mechanisms, such as immune complex formation [6] and T-cell mediated hypersensitivity,[7] may perhaps contribute to LLRs in some patients.

In our study, we observe for the first time that sera from a subset of patients with CLL who experience LLRs to mosquito bites, induces upregulation of CD63 on basophils from atopic donors. This is novel evidence that these sera contain factors which activate basophils. It provides indirect evidence of the presence of antibodies that activate basophils through the FcεRI. Given the clear association between autoantibody formation, autoimmune disease and CLL,[8] we believe they are most likely to be autoantibodies to the FcεRI,[13–15] and plan to characterize these by immunoblotting techniques in a future study.

Acknowledgments

Contribution: J.L., O.G.B. performed the experiments; J.L., O.G.B., S.L.F. analyzed the results and constructed the figures; S.P.M. conceived the concept; J.L., O.G.B., S.P.M. and S.L.F. developed and designed the research; J.L. wrote the paper.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at <http://dx.doi.org/10.3109/10428194.2016.1173208>.

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