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LETTER TO THE EDITOR

Evidence for CD19B–CD8T cell interactions in blood and tissues from patients with GvHD

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GvHD is a major complication of allogeneic hematopoietic stem cell transplantation (aHSCT), and is mediated by cytotoxic T cells (CTLs), recruited by chemokines such as CXCL9/MIG, CXCL10/IP-10 and CXCL11/ITAC to target organs, where they effect tissue injury.[1,2]

B-cell contribution to GvHD pathogenesis is widely accepted, but only partially explained. The role of B cells in the pathogenesis of immune responses is multifold, including Ab-mediated and -independent mechanisms, such as Ag presentation, and cytokine production.[3]

B cells from GvHD patients show altered homeostasis, conditioned by an unbalanced immune *milieu*, including abnormal B-cell activating factor (BAFF) concentrations, and leading to the differentiation of activated CD27 B cells spontaneously producing Igs.[4]

We described an interaction between autologous CD19 B cells and activated CD8 CTLs in normal donors.[5] *In vitro* Ag-stimulated CTLs form stable couplets with B cells independent of Ag presentation, through CD27 (on B cells)–CD70 (on T cells) axis. T-cell/B-cell coupling enhances CTL survival and proliferation, and stimulates cytokine release by B cells, including CXCL9/MIG, CXCL10/IP-10 and CXCL11/ITAC.

The CD27-70 interaction has a central role in B–T cross talk and activation[6–8] by promoting the development of the T-cell effector pool[9] and activating CTL-effector functions,[10] while contemporaneously inducing B-cell activation, expansion and differentiation into plasma cells.[11]

Interestingly, the pattern of cytokines secreted by B cells after coupling is consistent with their role in promoting migration of T cells to GvHD-affected organs.[1]

Here, we explored the interactions of B cells with CD8 cells in blood and tissues of GvHD patients.

We first performed a single-center study, retrospectively analyzing consecutive blood samples from 81 transplanted patients and 21 healthy controls, processed in the Central Hospital of Bolzano for immune-monitoring purposes (identification cohort). To comprehensively study the role of CD19–CD8 coupling in GvHD, we included patients with $>5\%$ of circulating CD19 cells, targeting therefore late acute and chronic forms, regardless of the ongoing therapy (details in Supplementary Tables 1S and 2S). The frequency of coupling was calculated as the percentage of viable $CD45^{++}/CD8^{++}/CD19^{+}$ cells (Figure 1a). Interestingly, the flow cytometry appearance of the couplet resembled a single $CD8^{++}/CD19^{+}$ event, whereas we already demonstrated that, by means of cell sorting, this cell population could be separated by fluid shear stress in single CD8 and CD19 cells.[5] Double-positive cells displayed consistently high forward scatter (FSC)/side scatter (SSC) scores (examples in Figure 1, Supplementary Figure 2S). Couplets stained negative for CD16, CD56, CD4 and positive for CD3 (data not shown).

We included samples collected only from patients with active GvHD, and a relatively recent onset, setting an arbitrary 150 days limit from onset. Non-GvHD controls included patients without previous or suspect GvHD.

As a further control, to rule out the influence of infection on coupling, we excluded patients and controls with known active viral and bacterial infections at the time of sampling. However, the frequency of couplets was not significantly different among patients with and without infections and no GvHD (data not shown), suggesting that the coupling occurs independently. The final number of evaluable samples was 20 GvHD samples, 15 non-GvHD controls, 21 healthy controls (details and statistical methods in Supplementary Table 1S).

Seven patients in the identification cohort were monitored before and after GvHD onset. B–T couplets were significantly higher in patients after GvHD onset and overall in the GvHD patient cohort compared with aHSCT patients without GvHD and healthy controls (Figures 1b and c).

The GvHD cohort included a subgroup of patients with percentage of couplets above the median value. This subgroup was otherwise similar in clinical characteristics (transplant type, stem cell source, type and grade of GvHD) to the other GvHD patients, save for the time after GvHD onset: patients analyzed within 30 days from GvHD onset had higher rate of couplets (Figure 1c, red dots).

Nine patients in the aHSCT cohort received therapeutic donor lymphocyte infusions (DLIs) for disease relapse. Couplets were significantly higher after DLI (Figure 1d) regardless of the subsequent GvHD development and disease response.

DLIs are composed by mixed lymphocytes, including B cells. Hence, we tested whether coupling correlated with the quantity of circulating B cells. This was true only for coupling after DLI, not after GvHD, suggesting that in GvHD the development of couplets is enhanced over their stochastic occurrence observed in the presence of higher B-cell numbers owing to DLI (Figures 2a and b).

Our findings were validated prospectively in an independent patient cohort (verification cohort).

Thirty-four consecutive samples from 23 pediatric patients transplanted in S Matteo Hospital of Pavia, were stained with CD45, CD3, CD8 and CD19 Abs after ficoll enrichment. Analyses were performed on FSC/SSC viable $CD45^{+}CD19^{+}CD8^{++}$ cells. All patients had $\geqslant 50$ blood B cells/μL. Patients were categorized as previously described (Supplementary Table 1S). Because of the limited number of patients, we also analyzed the data at any time after GvHD onset. Both analyses confirmed significantly higher CD19–CD8 couplets in GvHD patients (Figures 2c and d). Again, the percentage of couplets in patients developing GvHD within 30 days from sampling was higher.

Eleven patients in both cohorts had longitudinal post-GvHD samples. The kinetics of couplets with time distinguished significantly the GvHD group ($P=0.01$, Supplementary Figure 1S).

We confirmed the coupling phenomenon in an additional small GvHD patient cohort in Würzburg University Hospital. In this independent analysis, thawed samples were analyzed for B–T activation markers, which resulted specifically enhanced in the couplet (Supplementary Figure 2S).

Within the limitation of this preliminary study, we analyzed several clinical variables like severity of GvHD, GvHD outcome,

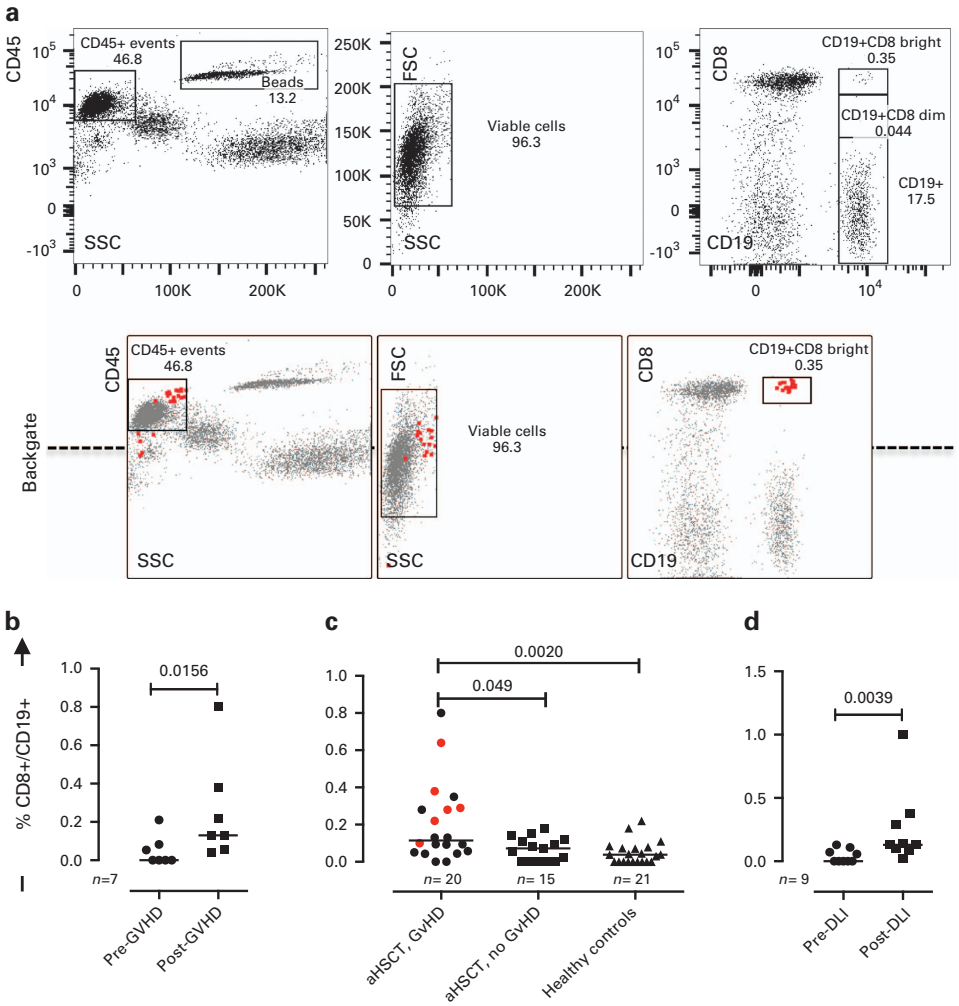

Figure 1. (**a**) Flow-cytometer analysis logical gating. Only couplets in the CD8 bright field are scored in the analysis. Back-gate row shows physical parameters of the CD8–CD19 cells in the regions. (**b**) Patients monitored before and after GvHD onset (median 33 days after GvHD onset: 15–116) measured with Wilcoxon matched-paired rank test. Median (min–max) values for the groups are: pre-GvHD 0 (0–0.21); post-GvHD 0.13 (0–0.38). (**c**) Couplets scoring in aHSCT patients and healthy controls, measured with Kruskal–Wallis unpaired test. Red dots are patients screened within 30 days after GvHD onset, black dots within 150 days. Median (min–max) values for the groups are: GvHD 0.115 (0–0.8); no GvHD 0.038 (0–0.22); healthy controls (HC) 0.072 (0–0.18). (**d**) Patients analyzed pre- and post DLI (median 29 days after DLI: 11–126) measured with Wilcoxon matched-paired rank test. Median (min–max) values for the groups are: pre-DLI 0 (0–0.13); post-DLI 0.13 (0.025–1).

ongoing therapy, but no relevant association other than time after GvHD onset emerged in the cohorts (Supplementary Table 2S).

Finally, we analyzed the presence and tissue distribution of B–T couplets in skin and gastrointestinal (GI) biopsies of GvHD tissues.

Thirteen GvHD samples from 12 aHSCT patients (4 GI and 9 skin biopsies) and 8 from controls (3 GI and 5 skin biopsies), including 6 aHSCT patients without GvHD and 2 healthy controls, were collected in Bolzano Hospital and analyzed by immunohistochemistry (IHC), or laser scanner microscopy (LSM; Supplementary Table 3S).[12]

B cells are usually absent in normal skin and very rare in GvHD skin, as other groups failed to find evidence of them.[13] By including also the slides with low numbers of B cells (1–10), we visualized CD8–CD19/20 couplets in skin and GI GvHD biopsies. Two independent analyses with different imaging platforms (IHC and LSM) confirmed higher couplets number in GvHD patients (Figure 2e, Supplementary Table 3S).

CD19–CD8 coupling is an intriguing phenomenon that we characterized in depth in Flu-Ag-specific CTLs expanded *in vitro*, demonstrating that it stimulates a pro-inflammatory interaction through CD27–CD70 contact and through secretion of soluble molecules.

This preliminary study aimed to verify the relevance of this *in vitro* observation in GvHD. The study captured data collected for clinical immune-monitoring purposes and only a limited immunophenotyping of the couplets was feasible. Conceivably, based on our preliminary data, the couplet is formed by donor-derived CD27+ B cells and CD70+ CTLs, but this has to be confirmed with deep phenotyping and lineage-specific chimerism analyses in a future larger prospective study. This future study could also better address immunologic variables potentially connected with coupling, as response to GvHD therapy or relapse.

The frequency of couplets observed *in vivo* rarely exceeds 0.5% of circulating CD45 lymphocytes. However, it is reproducibly and specifically detectable in GvHD patients early after diagnosis and regardless of therapy. Three patient cohorts independently analyzed with different methods (fresh blood, ficoll enrichment, thawed cells) demonstrated significantly the presence of couplets. This reproducibility could suggest the use of coupling as a GvHD biomarker.

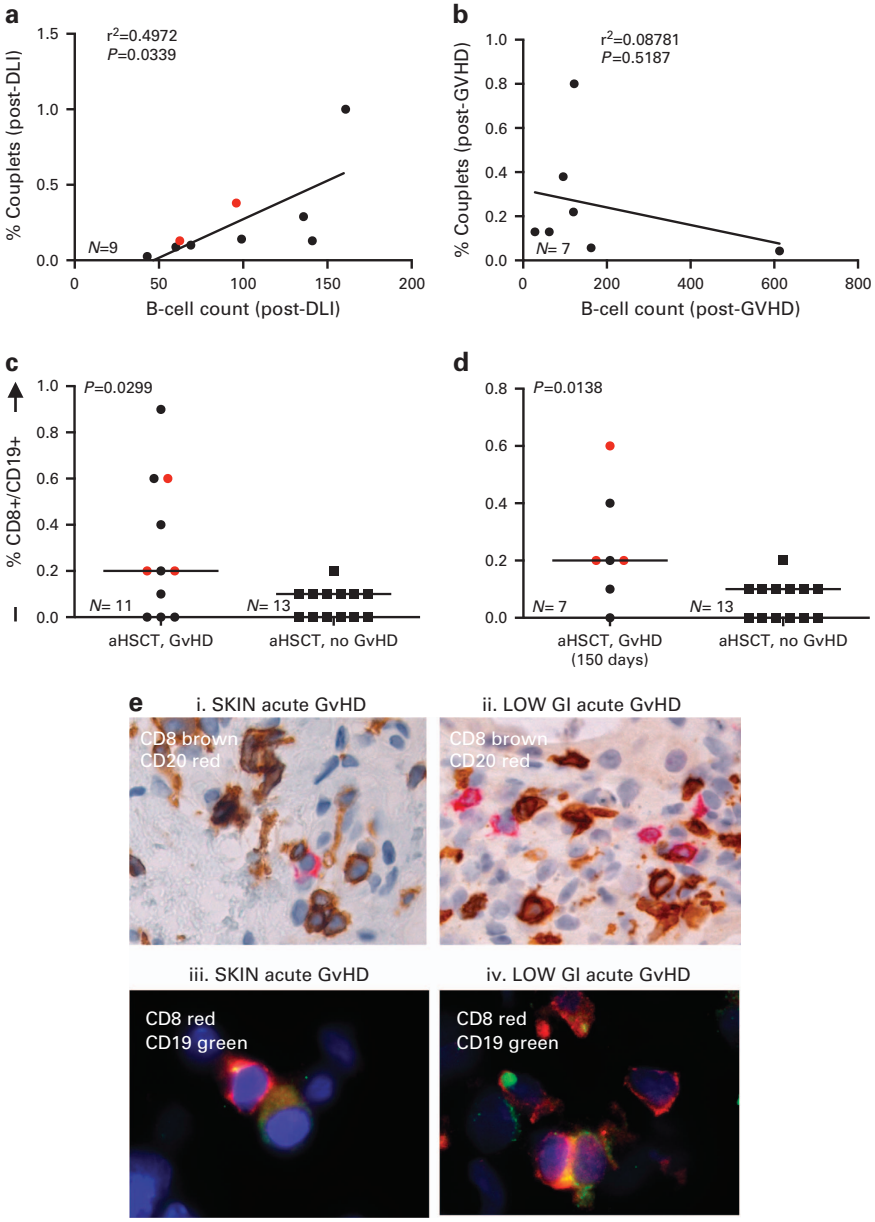

Figure 2. Linear regression analysis correlating number of peripheral blood B cells, and % of couplets in patients post DLI (**a**), and post-GvHD onset (**b**) identification patient set. Red dots are patients who developed GvHD post DLI. Same analyses correlating CD8 baseline cells did not achieve significance ($P = 0.617$ and $r^2 = 0.038$ post DLI, and $P = 0.579$, $r^2 = 0.064$ post GvHD). (**c** and **d**) Couplet scoring in aHSCT pediatric patients verification population, measured with Mann–Whitney unpaired tests. Black dots include all GvHD patients (**c**) and patients analyzed within 150 days after GvHD (**d**). Red dots are patients screened for couplets within 30 days after GvHD onset. Median (min–max) values for the groups are: 0.2 (0–0.9) and 0.2 (0–0.6) for GvHD patients in **c** and **d**, respectively; 0.1 (0–0.2) for both no-GvHD controls. (**e**) IHC staining on paraffine-embedded GvHD tissues (i, ii), and LSM on deparaffinized samples (iii, iv) showing examples of CD8 and CD19 coupling in GvHD tissues. See Supplementary Table 2S for more data.

Coupling was also present after DLI and correlated with a number of circulating B cells, suggesting that this is a relevant substrate for these interactions that are likely more stable, and frequent when T or B cells are in an active functional status as in the case of GvHD.

One limitation of the study is the lack of imaging of the couplet in flow cytometry analyses.

We rely on *bona fide* couplets after verifying high FSC–SSC parameters and after demonstrating in our previous work the sorting-based physical separation of the couplet.[5]

We cannot rule out membrane sharing between the two cell types after contact, and therefore the risk of overestimation by counting as a couplet a single cell that acquired the marker of the other lineage. In this case, the data would rather reflect a significant difference in coupling dynamics in GvHD, and still be valuable as a biomarker.

Tissue biopsies showed evidence of coupling, also involving limited amounts of cells.

As coupling is a transient phenomenon, a single time point sampling and tissue biopsy may not adequately describe a dynamic interaction. When we described *in vitro* CD19–CD8 coupling, we performed overnight time-lapse analyses showing that T cells engaged prolonged and multiple contacts with rather static B cells (http://www.jimmunol.org/content/180/3/1362/suppl/DC1).

A GvHD animal model could be more suitable for such *in vivo* investigation, also facilitating couplet analysis in lymphoid tissues, where similar interactions may take place.

We speculate that a cross talk between CD19 and CD8 lymphocytes triggers immunologic responses relevant to GvHD. This deserves further investigation and validation in a prospective study, specifically designed to capture the immunological trigger behind the coupling and the fate of the cells after this interaction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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S Deola[1,2], C Cugno[1,2,3], P Comoli[4], I Guido[4], L Rubert[3], M Zecca[3], D Zanolini[5], A Follenzi[5], M Langes[6], G Negri[7], M Tauber[7], I Pusceddu[6], I Cavattoni[2], M Svaldi[6], GU Grigoleit[8], E Baumeister[8], E Wang[1], S Rutella[1] and FM Marincola[1]

[1]Research Department, Division of Translational Medicine, Sidra Medical and Research Center, Doha, Qatar;
[2]Department of Hematology and Bone Marrow Transplantation, Ospedale Centrale, Bolzano, Bolzano, Italy;
[3]Department of Pediatric Hematology/Oncology, Fondazione IRCSS Policlinico San Matteo, Pavia, Italy;
[4]Pediatric Hematology/Oncology Laboratory, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;
[5]Department of Health Sciences, Università del Piemonte Orientale, Novara, Italy;
[6]Laboratory of Hematology, Ospedale Centrale, Bolzano, Italy;
[7]Department of Pathology, Ospedale Centrale, Bolzano, Italy and
[8]Department of Hematology and Oncology, University Hospital, Würzburg, Germany
E-mail: sdeola@sidra.org

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Supplementary Information accompanies this paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)