## APRIL levels are associated with disease activity in human chronic graft-*versus*-host disease

After allogeneic hematopoietic stem cell transplantation (AHSCT), acute GVHD is almost entirely mediated by donor T cells whereas chronic graft-*versus*-host disease (cGVHD) also involves donor B cells.[1] A breakdown in peripheral B cell homeostasis in cGVHD is in part due to high levels of B cell activating factor (BAFF), a member of the tumor necrosis factor (TNF) superfamily.[2] BAFF levels are increased in cGVHD and high BAFF/B-cell ratios are associated with cGVHD activity.[3] BAFF interacts with BAFF receptor (BAFF-R), B cell maturation antigen (BCMA) and transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI).[2] BAFF is involved in the pathogenesis of autoimmune diseases in which excess BAFF levels increase autoreactive B cells survival driving autoimmunity.[4] A proliferation-inducing ligand (APRIL) is another TNF ligand superfamily member acting with BAFF in the BAFF/APRIL system.[5] APRIL shares two receptors with BAFF (TACI and BCMA). APRIL is important in antibody class switching and plasma cells survival and is involved in the late stage of B cell differentiation.[5] Treatments targeting the BAFF/APRIL system or its receptors have been developed.[4] We assessed circulating APRIL levels, B cell surface BAFF/APRIL receptors expression and other B cell related parameters in a prospective cohort of AHSCT patients. We found that BAFF and APRIL levels were both increased in cGVHD patients compared with patients who did not develop cGVHD and healthy controls. APRIL levels, but not BAFF levels, were higher in active cGVHD patients compared with inactive cGVHD patients ($P$=0.006). APRIL levels were correlated with plasmablast frequencies in the cGVHD subgroup, and high APRIL levels were associated with antinuclear autoantibody production ($P$=0.01) and severe cGVHD ($P$=0.005).

This prospective study was conducted at Saint-Louis Hospital (Paris, France) from November 2010 to May 2015. Diagnosis, activity and staging of cGVHD were made using the National Institutes of Health (NIH) criteria.[6] Patients with active cGVHD were included at the time of their first cGVHD flare before starting cGVHD treatment, or at any time of cGVHD progression under cGVHD treatment. Progression was defined by an increase of at least 1 point in the NIH global severity score. Inactive cGVHD patients were included if they had partial response, defined as a decrease of at least 1 point in the NIH global severity score, or complete response. Patient characteristics are detailed in Table 1. All patients signed a written informed consent form in accordance with the recommendations of our local ethics committee.

Serum BAFF and APRIL levels were quantified using

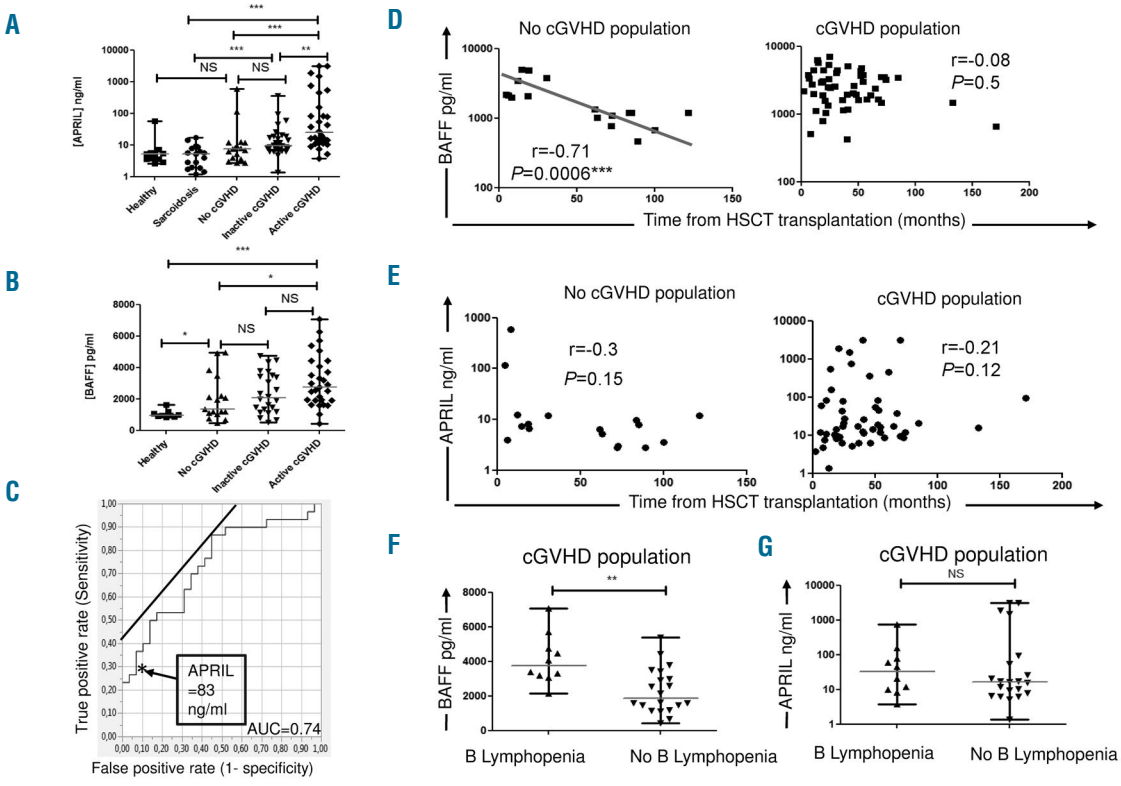


**Figure 1. BAFF and APRIL circulating levels associations with cGVHD activity, time from HSCT and B cell lymphopenia.** Circulating APRIL (A) and BAFF (B) levels were measured using an ELISA assay in cGVHD patients (n=31), inactive cGVHD patients (n=26) and patients without cGVHD (no cGVHD, n=16). (C). Receiver operative characteristic (ROC) curve of APRIL levels among active and inactive cGVHD patients. Area under the curve=0.74. (D). Correlations between BAFF circulating levels and time from HSCT in cGVHD and no cGVHD patients. (E). Correlations between APRIL circulating levels and time from HSCT in cGVHD and no cGVHD patients. Circulating BAFF (F) and APRIL levels (G) between patients with and without B-cell lymphopenia. Correlations were measured using the Spearman test: * $P$<0.05, ** $P$<0.01, *** $P$<0.001, NS: Non-significant.

**Table 1.** Characteristics of the 73 patients who underwent hematopoietic stem cell transplantation.

| Characteristics | Active cGVHD n=31 | Inactive cGVHD n=26 | No GVHD n=16 |
|---|---|---|---|
| Male n (%) | 19 (61) | 13 (50) | 5 (31) |
| Age at study inclusion y, median (range) | 47 (22-62) | 48 (21-67) | 54 (15-70) |
| Time from AHSCT in months, median (range) | 29.6 (2-99) | 45.2 (4.8-170.5) | 46 (4.6-121.7) |
| Hematological disease, n (%) | | | |
| Hodgkin/ NHL/ CLL/ MM | 2 (6)/7 (23)/0/2 (6) | 1(4)/2(8)/2(8)/4(15) | 0/0/1 (6)/0 |
| AML/ ALL | 7 (23)/4 (13) | 6 (23)/4 (15) | 3 (19)/2 (12) |
| MDS/ MPS | 5 (17)/4 (13) | 3 (12)/3 (12) | 3 (19)/3 (19) |
| Aplastic anemia | 0 | 0 | 1 (6) |
| Hemoglobinopathy | 0 (0) | 1 (4) | 3 (19) |
| Conditioning: | | | |
| Myeloablative n (%) | 14 (45) | 13 (50) | 9 (56) |
| Nonmyeloablative n (%) | 17 (55) | 13 (50) | 7 (44) |
| HLA Matching (n %) | | | |
| Matched, unrelated | 17 (54) | 13 (50) | 8 (50) |
| Matched, related | 10 (32) | 11 (42) | 6 (37) |
| Mismatched | 4 (13) | 2 (8) | 2 (13) |
| cGVHD organ involvement n (%) | | | |
| Skin | 29 (93) | 23 (88) | |
| Lichenoid | 15 (48) | 8 (31) | |
| Sclerodermiform | 14 (45) | 13(50) | |
| Ocular | 7 (23) | 8 (31) | |
| Oral mucosa | 15 (48) | 8 (31) | |
| Lung | 10 (32) | 6 (23) | |
| Digestive tract | 5 (17) | 2 (8) | |
| Liver | 12 (38) | 1 (4) | |
| cGVHD NIH severity n (%) | | | |
| Mild-moderate | 11 (35) | 18 (69) | |
| Severe | 20 (65) | 8 (31) | |
| Immunosuppressive treatment n (%) | | | |
| Systemic corticosteroids | 24 (77) | 18 (72) | 2 (12.5) |
| Ciclosporin/ Tacrolimus | 6 (20)/0 | 4 (12)/0 | 2 (12.5)/0 |
| MMF/ MTX | 4 (13)/ 1 (3) | 2 (8)/0 | 0 |
| Rapamycin/ Everolimus | 1 (3/2(7) | 0/2 (8) | 0 |
| Imatinib | 1 (3) | 1 (4) | 0 |

*AHSCT: allogeneic hematopoietic stem cell transplantation; cGVHD: chronic graft-versus-host disease; NHL: non-Hodgkin lymphoma; CLL: chronic lymphocytic leukemia; MM: multiple myeloma; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MDS: myelodysplastic syndrome; MPS: myeloproliferative syndrome; MMF: mycophenolate mofetil; MTX: methotrexate.*

the human BAFF/Blys Quantikine H ELISA immunoassay (R&D Systems) and the Human APRIL Platinum ELISA assay (Affymetrix eBioscience). Considering the sensitivity of the test, the minimum detectable dose was 2.68 pg/ml (range 1.01-6.44 pg/ml) in the first test and 0.007 ng/ml (range 0.003-0.015 ng/ml) in the second, according to the manufacturers' instructions. Total peripheral blood mononuclear cells from cGVHD patients, HSCT recipients with no cGVHD and healthy controls were stained with fluorochrome conjugated antibodies (*Online Supplementary Table S1*) to analyze B cell expression of BAFF-R, TACI and BCMA and separate B cell populations: transitional $CD24^{high}CD38^{high}CD27^{-}$, naive $CD24^{+/low}CD38^{+/low}CD27^{-}$, memory $CD24^{high}CD38^{neg}CD27+$ B cells and $CD24^{-}CD27^{high}CD38^{high}$ plasmablasts.[7–9] Data are presented as median (range) or counts (percentage).

A Chi-squared test and Fisher's exact test were used to compare qualitative variables; a Wilcoxon or a Mann-Whitney test was used to compare paired or non-paired non-normally distributed variables, respectively. The Spearman test was used for correlations. Statistical significance was indicated: *$P$< 0.05 **$P$< 0.01; ***,$P$<0.001. Parameters with a $P$ value <0.2 in univariate analysis were entered in a multivariate logistic regression, with Y as the dependent variable.

Active cGVHD patients had significantly higher APRIL levels compared with inactive cGVHD patients: 24.18 ng/ml [3.82-3896] *vs.* 11.75 ng/ml [5.73-356] ($P$=0.006) (Figure 1A). Active cGVHD patients had significantly higher APRIL levels compared with no cGVHD ($P$=0.0005), healthy controls ($P$<0.0001) and sarcoidosis patients ($P$<0.0001) used as a control group (sarcoidosis is an inflammatory granulomatous disease displaying high BAFF levels).[10] Considering active and inactive cGVHD using a Receiver operating characteristic (ROC) curve (Figure 1C), we found that an APRIL level of 83 ng/ml was associated with active cGVHD with a specificity of 93.1% and sensitivity of 33%. Active cGVHD patients had higher BAFF levels compared with no cGVHD patients ($P$=0.03) and healthy controls ($P$<0.001). There was no significant difference of BAFF levels between

active and inactive cGVHD (Figure 1B). BAFF levels were negatively correlated with time from AHSCT (r=-0.71; *P*=0.006) in the no cGVHD group, but not in the cGVHD group because of persistent elevated BAFF levels (Figure 1D). BAFF levels were higher in patients with B cell lymphopenia: 3727 pg/ml [1966-7048], compared with patients without B cell lymphopenia: 2088 pg/ml [419-5383], (*P*=0.0021) (Figure 1F). There was no association between time from AHSCT or B cell lymphopenia and APRIL circulating levels (r=-0.3; P=0.15; and *P*=0.59, respectively) (Figure 1E,G). In the no cGVHD population BAFF levels were negatively correlated with memory B cell frequencies (r=-0.72; *P*=0.002) (*Online Supplementary Figure S1*). In the cGVHD population APRIL levels were positively correlated with plasmablast frequencies (r=+0.32, *P*=0.04) (*Online Supplementary Figure S1*). There was no difference in the circulating levels of BAFF and APRIL in the antinuclear antibodies (ANA) + *versus* the ANA- group but patients with high APRIL levels (> 83ng/ml) were more frequently positive for ANA than patients with low APRIL levels: 100% *versus* 60% (*P*=0.01) (*Online Supplementary Figure S1*). Moreover, in univariate analysis, high APRIL levels were associated with severe cGVHD (*P*=0.005) and pulmonary involvement (*P*=0.02). There was no association with the involvement of other organs or treatments received, such as systemic corticosteroids or other immunosuppressive therapies. In multivariate analysis, high APRIL levels were only associated with severe cGVHD (*P*=0.03) (*Online Supplementary Table S2*). We determined the level of cell surface expression of BAFF-R, TACI and BCMA on B cells in each group. The frequencies of BAFF-R positive B cells were decreased in active cGVHD compared with inactive cGVHD (*P*=0.01), no cGVHD patients (*P*=0.03) and healthy controls (*P*=0.01) (Figure 2). Considering BAFF-R MFI on B cells, there was a significant decrease in BAFF-R expression between cGVHD and no cGVHD patients (*P*=0.03). The frequencies of TACI positive B cells were increased in active cGVHD compared with no cGVHD (*P*=0.03) but not compared with inactive cGVHD patients (*P*=0.08). The frequencies of BCMA positive B cells were increased in active cGVHD compared with no cGVHD (*P*=0.03) but not compared with inactive cGVHD patients (*P*=0.12).

Our results are in accordance with recent findings suggesting that after allogeneic HSCT, in the context of B cell lymphopenia, persistently elevated BAFF levels may promote cGVHD by attenuating BCR-triggered apoptosis of polyreactive B cells.[1,11] In contrast, patients without cGVHD had efficient recovery of BAFF receptors-expressing B cells, which are able to sequester BAFF and therefore prevent the promotion of auto-reactive B cells.[12,13] This model did not take into account the possible role of APRIL in disturbed B cell homeostasis during cGVHD. We found that circulating APRIL levels were significantly higher in active cGVHD compared with inactive cGVHD patients. However, as previously reported,[3] BAFF circulating levels were not associated with cGVHD activity . The role of APRIL as a biomarker of activity in autoimmune diseases such as SLE remains conflictual,[4] however our

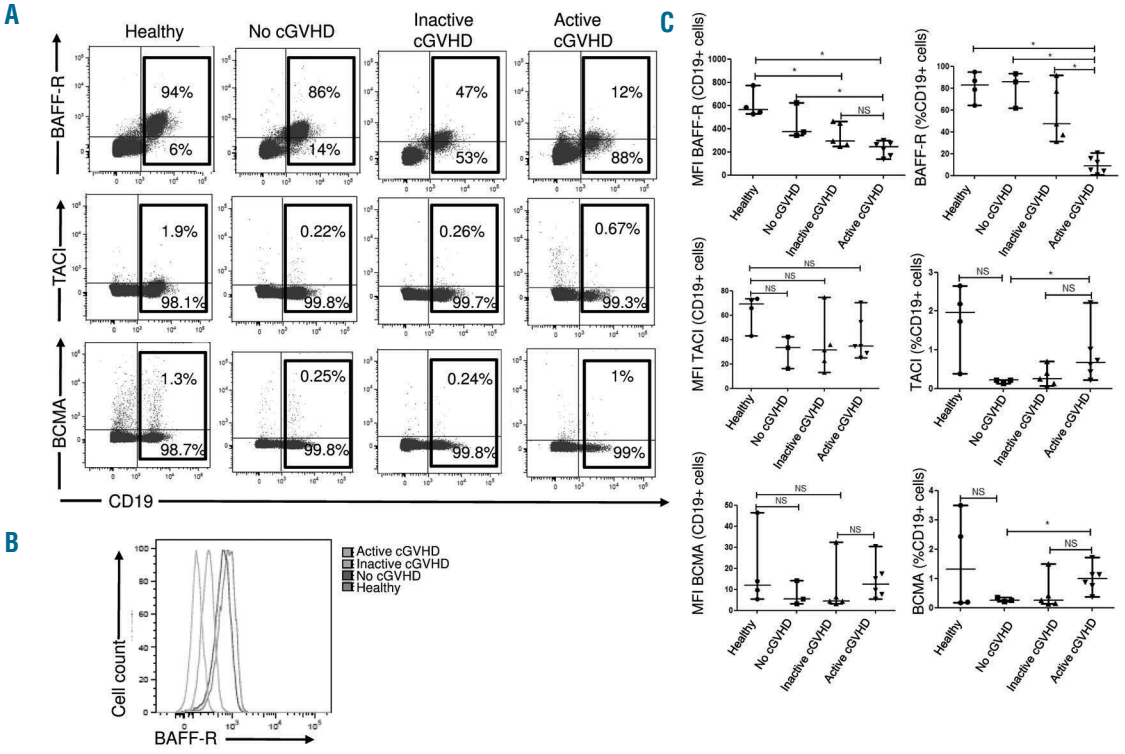


**Figure 2. BAFF-R, TACI and BCMA expression on CD19⁺ B-cells among hematopoietic stem cell transplant recipients and healthy controls.** (A). Representative dot plots of BAFF-R, TACI and BCMA expression among CD19⁺ B cells from active, inactive, no cGVHD patients and healthy controls. (B). Representative median fluorescence intensity (MFI) expression of BAFF-R among active, inactive, no cGVHD patients and healthy controls. (C). Median frequency and MFI of BAFF-R, TACI and BCMA expression among CD19⁺ B cells from active (n=6), inactive (n=5); no cGVHD (n=3) patients and healthy controls (n=4). **P*<0.05, ***P*<0.01, ****P*<0.001, NS: Non-significant.

results suggest that APRIL may be a biomarker of cGVHD activity. Nevertheless, some patients with active cGVHD had low APRIL levels comparable with healthy controls or with patients without cGVHD. In current clinical practice, the use of APRIL as a potential biomarker of activity would be interesting with APRIL levels associated with a low false positive rate. We therefore determined that high APRIL levels of > 83 ng/ml were associated with a specificity of 93.1% and sensitivity of 33%. Interestingly, patients with high APRIL levels had an increased frequency of severe cGVHD, ANA production and pulmonary involvement. Nevertheless, these results should be confirmed by a validation cohort to determine the predictive positive value of the threshold of 83ng/ml.

It is of note that we found a positive correlation between circulating APRIL levels and plasmablast frequencies in cGVHD patients. These results are consistent with the role of APRIL in the late stages of B cell differentiation.[4,5] The quantitative level of expression of each receptor has been shown to be a key factor in B cell homeostasis.[14,15] We found that active cGVHD patients had a decreased expression of BAFF-R and an increased expression of BCMA and TACI, as previously found in 2 cGVHD patients.[3] Decreased expression of BAFF-R and parallel increased expression of BCMA have been associated with the differentiation of B-cells into immunoglobulin-secreting cells and plasmablasts.[15] APRIL is able to bind strongly to BCMA[4] and the increased APRIL levels observed in cGVHD may promote plasmablast differentiation, survival and autoantibody production.[5]

Our results suggest that circulating APRIL may be a hallmark of active cGVHD and may play a role in the late stage of B-cell homeostasis such as plasmablastic maturation and autoantibody production.

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