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Neutrophil extracellular traps, endothelial injury and use of abatacept for graft *versus* host disease prophylaxis

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Main finding: Our findings suggest broader clinical benefits of abatacept than previously described with potential reduction in endothelial injury in addition to acute GVHD reduction.

Running Head: Abatacept and endothelial injury

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The early post-transplant period is crucial for patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT), as the foundation for later complications resulting from early endothelial injury is laid in this time. Interventions during this period can play a defining role in outcomes of HSCT. Abatacept is a recombinant T-cell co-stimulation blockade agent shown to be effective in reducing acute graft versus host disease (aGVHD).[1-3] Abatacept improves not only inflammatory disease but also cardiovascular and pulmonary outcomes in patients with rheumatologic disorders, perhaps suggesting activities broader than T-cell inhibition.[4-6] Neutrophil extracellular traps (NETs) consist of decondensed chromatin surrounded by histone and granular proteins like myeloperoxidase (MPO) and neutrophil elastase (NE). [7] Dysregulation of NET production (NETosis) has been shown to play a role in endothelial injury. [8-10] We propose that abatacept not only decreases the risk of aGVHD but may also reduce the incidence of endothelial injury syndromes like engraftment syndrome, transplant associated thrombotic microangiopathy (TA-TMA), and lung injury, by limiting NETosis and complement activation.

We collected plasma samples from 262 consecutive pediatric HSCT patients who underwent allogeneic HSCT at Cincinnati Children's Hospital Medical Center (CCHMC) and consented to participate in the CCHMC HSCT biorepository, approved by the institutional review board. We reviewed medical records and clinical databases for demographics and clinical outcomes. Patients were divided into three main categories regarding their GVHD prophylaxis - patients who received calcineurin inhibitor-based therapy with either mycophenolic acid (MMF) or methotrexate (CNI), abatacept in addition to CNI based therapy with either MMF or methotrexate (CNI-ABA), and patients who received an ex vivo T-cell depleted graft (TCD). Abatacept was given per recommended guidelines on days -1, +5, +14, and + 28 at 10mg/kg per dose.[1-3] All patients were prospectively screened for TA-TMA pre-transplantation baseline and risk stratified using Jodele criteria[11]. GVHD was graded using The Glucksberg criteria.[12]

Engraftment syndrome was diagnosed using CIBMTR criteria. We recorded any supplemental oxygen requirement for more than 1 hour, with particular attention to the peri-engraftment (day +7 to +21) period as a marker of early pulmonary injury.

Patient demographics are shown in Supplemental Table 1. Fifty-three percent of patients (n=140) received CNI GVHD prophylaxis without abatacept, 26% (n=48) received CNI-ABA and 28% a TCD graft without additional immune suppression (n=74). Thirty-three percent of HSCT recipients (n=87) required supplemental oxygen in the early transplant period (from start of conditioning to day +26 (simple mask/blow by: 25% (n= 22/87), nasal cannula: 53% (n=46/87), non-invasive pressure support: 3% (n=7/87), mechanical ventilation: 14% (n=12/87)), at a median of day +8 (range -13, +26), for a median of 4 days (range: 1-57). TA-TMA was diagnosed at a median of 25.5 days (IQR: 15-48 days) after HSCT.

We measured day +14 dsDNA levels as a surrogate for NETosis using the Quant-iT PicoGreen dsDNA reagent and kit, as previously described. [13] Patients who received CNI-ABA GVHD prophylaxis had lower median day +14 dsDNA compared to patients who received CNI or TCD (126 ng/mL vs. 165 ng/mL vs 170 ng/mL, $p<0.0001$) (Figure 1a). Underlying diagnosis, stem cell source, conditioning regimen, and GVHD prophylaxis all significantly modified dsDNA levels in univariate analysis (Supplemental Table S1). We and others have shown that NETosis activates complement, leading to endothelial injury and TA-TMA. [10, 13, 14] sC5b9 levels are used clinically as a marker of complement activation. Patients who received CNI-ABA had a lower median sC5b-9 level compared to patients who received CNI or TCD (123 ng/mL vs 144 ng/mL vs 151 ng/mL, $p=0.005$) (Figure 1b). We also measured TGFβ levels after observing the effect of abatacept on NETosis to see if reduced endothelial injury and complement activation could be related to the effect of abatacept on residual host antigen presenting cells like macrophages. TGFβ at day 14 was significantly lower in patients who received CNI-ABA compared to CNI or TCD (369 ng/mL vs 598 ng/mL vs 833 ng/mL $p=0.001$).

Patients who received CNI-ABA were significantly less likely to develop peri-engraftment supplemental oxygen requirement, engraftment syndrome (ES), and TA-TMA in univariate analysis (Table 2). Patients who received CNI-ABA had a lower incidence of acute GVHD ≥ grade 2 compared to the CNI group, similar to the TCD group (Table 2). There were no statistical differences in 1-year overall mortality and non-relapse mortality (Table 2). We then performed a multivariate analysis adjusted for diagnosis (malignancy/no malignancy) and conditioning regimen intensity (MAC/RIC) using CNI-ABA recipients as the reference group. The odds ratios (OR) for peri-engraftment oxygen requirement (CNI OR: 3.78 (CI 95%: 1.65-9.83, p=0.003, TCD OR: 3.15 (CI 95%: 1.27-8.68, p=0.018) and ES (CNI OR: 3.43, CI 95%: 1.23-12.24, p=0.031, TCD OR: 5.53, CI 95%: 1.86-20.7, p= 0.004) were significantly higher for patients who received CNI and TCD. The OR for TA-TMA was higher in patients who received CNI (CNI OR: 2.57, CI 95%: 1.2-5.90, p=0.019) but similar in TCD and CNI-ABA recipients (TCD OR: 1.31, CI 95%: 0.55-3.28, p=0.55).

Abatacept limits T cell activity so we looked at viremia within 1 year from transplant. We found a higher frequency of EBV viremia in patients who received CNI-ABA both in univariate analysis (Supplemental Table S2) as well as multivariate analysis when CNI-ABA used as the reference group (CNI OR: 0.48, CI 95%: 0.24-0.95, p=0.04, TCD OR: 0.32, CI 95%: 0.14-0.68, p=0.003). However, the number of patients who received EBV-directed treatment was similar in each group, suggesting EBV reactivation was mild (Supplemental Table S2). None of the patients with EBV reactivation in CNI-ABA group developed idiopathic pneumonia syndrome. There was no statistical difference in rate of CMV reactivation in CNI-ABA group compared to other groups in a univariate analysis (Supplemental Table S2). However, in multivariate analysis adjusted for age and diagnosis, patients who received CNI and TCD had lower risk of CMV viremia than CNI-ABA group (CNI OR: 0.45, CI 95%: 0.22-0.92, p=0.03, TCD OR: 0.45, CI 95%: 0.2-1, p=0.05). Patients who received CNI-ABA had lower rates of BK viremia compared to CNI and

TCD groups (Supplemental Table S2) in univariate analysis. The TCD recipients had a higher OR for BK viremia compared to the CNI-ABA and CNI recipients in multivariate analysis (TCD OR: 3.68, CI 95%: 1.63-8.83, p=0.002, CNI OR: 2.05, CI 95%: 0.97- 4.61, p=0.07). The rates of BK cystitis were not statistically different between the 3 groups (Supplemental Table S2). Patients who received CNI-ABA and CNI regimens had lower rates of adenovirus reactivation than patients who received TCD graft in univariate (Supplemental Table S2) but not in multivariate analysis (CNI OR: 3.42, CI 95%: 1.12-14.98, p=0.06; TCD OR: 1.83, CI 95%: 0.49-8.83, p=0.4). Patients who received CNI-ABA had fewer bloodstream infections in the first 100 days, compared to other groups (Supplemental Table S2).

The significant decrease in NETosis we observed in patients who received CNI-ABA made us consider the hypothesis that abatacept may have a direct effect on neutrophils. We analyzed RNA expression in neutrophils isolated from day 14 blood in an exploratory study. We compared 3 patients who received a CNI based regimen to 3 patients who received CNI-ABA (Supplemental Table 1C). This preliminary analysis suggested potentially important effect of abatacept on neutrophils. Thirty genes were differentially expressed in neutrophils of patients who received CNI-ABA compared to patients who did not (Supplemental Figure 1A). MHC class II molecules, HLA-DQB1 and HLA-DRB5 showed reduced expression in neutrophils of abatacept recipients, suggesting reduced neutrophil activation. Gene set enrichment pathway analysis using Enrichr (KEGG2021 pathway library) indicated important changes in pathways associated with inflammatory processes, including asthma, antigen presentation, GVHD, T cell differentiation, and ferroptosis, suggesting possible biologically important modifications of neutrophil function in addition to T cells (Supplemental Figure 1B). [15] In a separate experiment, we measured dsDNA levels (ng/mL) in the supernatant of ex-vivo neutrophils stimulated with phorbol myristate acetate (PMA) with and without abatacept (Supplemental Figure 1D). Neutrophils were isolated (Mitenyi, 130-104-434) from a healthy control and 4 baseline patient

samples and incubated with and without 300 ug/mL abatacept for 1 hour at 37°C before stimulation with 50 nM PMA at 37°C for 4 hours. The addition of abatacept did not decrease NETs formation as measured by dsDNA in the culture supernatant. These preliminary results suggest that abatacept likely does not directly modulate PMA-induced NETosis in-vitro. Effects of abatacept on NETosis observed in-vivo are likely mediated through more complex immunologic interactions that cannot be recapitulated in a simplified PMA-driven in-vitro system. Further targeted studies are in process to understand this complicated balance that takes place in nascent neutrophils in the setting of HSCT and various types of GVHD prophylaxis. Taken together, our results show that abatacept may have broad benefits in patients undergoing allogeneic HSCT, and this effect may be associated with decreased NETosis and complement activation. Further prospective studies are needed to assess the role of abatacept in patients at risk for endothelial and pulmonary injury.

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Tables:

Table 1: Demographics according to Graft *Versus* Host Disease prophylaxis

	CNI based (n=140)	CNI based with Abatacept (n=48)	T cell-depletion (n=74)	p
Age at transplant (years, median)	9.03	6.87	9.57	0.07
Gender (M) (n)	83 (59%)	27 (56%)	42 (57%)	0.9
Race				0.034
Caucasian	131 (94%)	45 (94%)	62 (84%)	
AA	6 (4%)	2 (6%)	11 (16%)	
Diagnosis				<0.001
Malignancy, n (%)	47 (34%)	17 (35%)	9 (12%)	
Immune deficiency, n (%)	43 (31%)	0	16 (22%)	
Marrow failure, n (%)	25 (18%)	2 (4%)	47 (64%)	
Hematologic disorder, n (%)	14 (10%)	27 (56%)	2 (2%)	
Metabolic disorder, n (%)	11 (7%)	2 (4%)	0	
Donor type				0.04
Unrelated, n (%)	90 (64%)	30 (63%)	56 (76%)	
Related, n (%)	50 (36%)	18 (38%)	18 (24%)	
HLA type				0.001
Matched, n (%)	110 (79%)	36 (75%)	40 (54%)	
Mismatched, n (%)	30 (21%)	12 (25%)	34 (46%)	
Stem cell source				<0.001
BM, n (%)	114 (81%)	44 (92%)	4 (5%)	
PBSC, n (%)	13 (9%)	3 (6%)	70 (95%)	
Cord, n (%)	13 (9%)	1 (2%)		
Conditioning regimen type				<0.001
MAC, n (%)	77 (55%)	41 (85%)	47 (64%)	
RIC, n (%)	63 (45%)	7 (15%)	27 (36%)	

*AA: African American; BM: Bone Marrow; PBSC: Peripheral Blood Stem Cell; MAC: Myeloablative conditioning; RIC: Reduced Intensity Conditioning

Table 2: Graft Versus Host Disease prophylaxis and hematopoietic stem cell transplantation outcomes. Univariate analysis, (n, %)

	CNI based (n=140)	**CNI based with Abatacept (n=48)**	**T-depleted (n=74)**	**p**
Oxygen requirement	55 (39%)	7 (15%)	25 (34%)	0.005
Engraftment syndrome	29 (21%)	4 (8%)	20 (27%)	0.035
GVHD ≥ Grade 2	35 (25%)	1 (2%)	2 (3 %)	<0.001
TA-TMA	55 (39%)	10 (21%)	18 (24%)	0.018
Mortality at 1 year	18 (13%)	2 (4%)	10 (14%)	0.21
NRM at 1 year	13 (9%)	1 (2%)	10 (14%)	0.074
ANC >500 day (median, range)	13 (11-17)	12.5 (11-17)	10.5 (9-11)	<0.001

GVHD: Graft Versus Host Disease, TA-TMA: Transplant Associated Thrombotic Microangiopathy, ANC: Absolute Neutrophil Count.

Figure legends:

Figure 1. dsDNA and sc5b9 levels according to GVHD prophylaxis:

A. GVHD prophylaxis and day 14 dsDNA levels (median, IQR, (ng/mL)): y axis represents dsDNA levels, x axis represents GVHD prophylaxis – CNI (red dots), n=140, CNI-ABA (yellow dots) (n=48), TCD: (light blue dots) (n=78). Day 14 dsDNA levels were significantly lower in patients who received CNI-ABA for GVHD prophylaxis (164.8 vs 125.7 vs 169.5). **B.** GVHD prophylaxis and day 7 sC5b9 levels (median, IQR, (ng/mL)): y axis represents sc5b9 levels, x axis represents GVHD prophylaxis – CNI (red dots) (n=85), CNI-ABA (yellow dots) (n=48), TCD: (light blue dots) (n=67). Day 7 sc5b9 levels were significantly lower in patients who received CNI-ABA for GVHD prophylaxis (144 vs 123 vs 151). Unpaired samples were compared using Wilcoxon Rank Sum Test (equivalent to Mann-Whitney test).

A
<0.0001
<0.0001
dsDNA (ng/mL)
1500
1000
500
300
200
100
CNI
164.8 (133-219.9)
n=140
CNI-ABA
125.7 (98.08-162.8)
n=48
TCD
169.5 (125.8-298.2)
n=78

B
0.0034
0.003
sC5b-9 ng/mL
1500
1000
500
250
200
150
100
50
CNI
144 (110-201)
n=85
CNI-ABA
123 (87.5-158.3)
n=48
TCD
151 (103-192)
n=67

Supplemental table S1: Demographics and median day 14 dsDNA levels

	Allogeneic HSCT patients (N=262)	NETs (ng/mL, median)	p
Age at transplant (years, median)	8.57 (0-35)	160	
Gender			0.76
Female	110 (42%)	161.3	
Male	152 (58%)	158.9	
Race			0.14
Caucasian	237 (90%)	160.3	
African American	19 (7%)	188.4	
Asian	5 (2%)	150.6	
Hispanic	1 (1%)	410	
Diagnosis			0.0003
Malignancy, n (%)	74 (28%)	152	
Immune deficiency, n (%)	54 (21%)	174.4	
Marrow failure, n (%)	75 (29%)	187.7	
Hematologic disorder, n (%)	44 (18%)	135.1	
Metabolic disorder, n (%)	15 (6%)	125.7	
Donor type			0.34
Unrelated, n (%)	179 (68%)	162.3	
Related, n (%)	83 (32%)	154.3	
HLA type			0.62
Matched, n (%)	186 (71%)	160.8	
Mismatched, n (%)	76 (29%)	158.4	
Stem cell source			<0.0001
BM, n (%)	162 (62%)	155.1	
PBSC, n (%)	86 (33%)	181.4	
Cord, n (%)	14 (5%)	121.8	
Conditioning regimen type			0.0003
MAC, n (%)	177 (68%)	156.1	
RIC, n (%)	85 (32%)	162.7	
GVHD prophylaxis			<0.0001
CNI based	140 (53%)	164.8	
CNI based + abatacept	48 (18%)	125.4	
T cell depletion	74 (28%)	169.5	

*AA: African American; BM: Bone Marrow; PBSC: Peripheral Blood Stem Cell; MAC: Myeloablative conditioning; RIC: Reduced Intensity Conditioning

Supplemental Table S2. Bacterial blood stream infections within first 100 days, and viral reactivation within 1 year after HSCT according to GVHD prophylaxis

	CNI based (n=140)	CNI based with Abatacept (n=48)	T cell-depletion (n=74)	p
Viral reactivation within 1 year, n (%)				
EBV	63 (45%)	30 (63%)	25 (34%)	0.008
EBV viremia received treatment	25 (18%)	10 (21%)	16 (22%)	0.76
CMV	36 (26%)	20 (42%)	20 (27%)	0.11
ADV	28 (2%)	3 (6%)	8 (11%)	0.04
BKV	50 (36%)	11 (23%)	35 (47%)	0.02
BK Cystitis	23 (16.4%)	6 (12.5%)	20 (28%)	0.07
BSI in first 100 days, n (%)	36 (26%)	4 (8%)	14 (19%)	0.03

BSI: Bloodstream infection, any bacteriemia within 100 days. Positive viremia: Increased viral load more than two consecutive tests within 1 year from transplant (Ebstein-Barr Virus (EBV): >200 copies/mL, cytomegalovirus (CMV): >500 copies/mL, BK virus (BKV): >500 copies/mL, adenovirus (ADV): >500 copies/mL).

Supplemental Figure 1: Differential transcriptomics of day 14 neutrophils of patients who received abatacept and those who did not, with pathway analysis:

A

B

Pathways	p-value
Asthma	0.00098
Allograft rejection	0.00148
Graft-versus-host disease	0.0018
Type I diabetes mellitus	0.00189
Intestinal immune network for IgA production	0.00235
Autoimmune thyroid disease	0.00286
Epstein-Barr virus infection	0.00337
Mineral absorption	0.00365
Viral myocarditis	0.00365
Inflammatory bowel disease	0.00427
Leishmaniasis	0.00594
Antigen processing and presentation	0.00609
Th1 and Th2 cell differentiation	0.00837
Rheumatoid arthritis	0.00855
Staphylococcus aureus infection	0.00891
Hematopoietic cell lineage	0.00964
Th17 cell differentiation	0.01119
Toxoplasmosis	0.01221
Systemic lupus erythematosus	0.01739
Cell adhesion molecules	0.02066
Phagosome	0.02172
Influenza A	0.02732
Tuberculosis	0.02971
Human T-cell leukemia virus 1 infection	0.04247
RNA polymerase	0.0455
Ferroptosis	0.05975

C

#	Diagnosis	Age at HSCT (years)	Conditioning regimen	GVHD prophylaxis
1	Hemoglobin Hammersmith	20.25	Bu/Flu/TT	Abatacept/CNI/MMF
2	Beta-Thalassemia	2.08	Bu/Flu/TT	Abatacept/ CNI /MMF
3	SDS	2.43	Flu/Mel/Alemtuzumab	Abatacept/ CNI /MMF
4	Hurler syndrome	1.1	Bu/Cy/ATG	CNI /MMF
5	ALL	5.9	TBI/Cy	CNI /MMF
6	ALL	1.3	Carb/TT/Mel	CNI /MMF

D

Supplemental Figure 1: A. Heatmap of all the genes that are differentially expressed (NCBI accession number: 24929897): Red color is low expression, and green in high expression. First 3 patients (columns) (white heading) received CNI based regimen only without abatacept, and last 3 patients (columns) (blue heading) received abatacept as well. Expression of thirty genes were differentially regulated in the neutrophils of patients who received abatacept in addition to CNI based regimen. MHC class II molecules, HLA-DQB1 and HLA-DRB5 genes were upregulated in the neutrophils patients of who received CNI based regimen without abatacept compared with those receiving abatacept and a CNI-based regimen, suggesting decreased neutrophil activation in patients who received abatacept. **B.** Gene set enrichment pathway analysis using Enrichr (KEGG2021 pathway library) showing enrichment in pathways associated with inflammatory processes, including antigen presentation, T cell differentiation, and ferroptosis. **C.** Demographics of patients who had day +14 neutrophil isolation for RNA transcriptomics. **D.** dsDNA levels (ng/mL) in the supernatant of ex-vivo neutrophils stimulated with phorbol myristate acetate (PMA) with and without abatacept (Neutrophils were isolated (Mitenyi, 130-104-434) and incubated with and without 300 ug/mL abatacept for 1 hour at 37°C before stimulation with 50 nM PMA at 37°C for 4 hours