Tocilizumab, tacrolimus and methotrexate for the prevention of acute graft-versus-host disease: low incidence of lower gastrointestinal tract disease

William R. Drobyski,1 Aniko Szabo,2 Fenlu Zhu,2 Carolyn Keever-Taylor,1 Kyle M. Hebert,3 Renee Dunn,3 Sharon Yim,1 Bryon Johnson,1 Anita D’Souza,1 Mary Eapen,1 Timothy S. Fenske,1 Parameswaran Hari,1 Mehdi Hamadani,1 Mary M. Horowitz,1 J. Douglas Rizzo,1 Wael Saber,2 Nirav Shah,2 Bronwen Shaw1 and Marcelo Pasquini1

1The Department of Medicine; 2The Division of Biostatistics, Institute for Health and Society and 3The Center for International Blood and Marrow Transplant Research, Medical College of Wisconsin, Milwaukee, WI, USA

©2018 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2017.183434

Received: October 26, 2017.
Accepted: January 18, 2018.
Pre-published: January 19, 2018.
Correspondence: wdrobysk@mcw.edu
SUPPLEMENTAL METHODS

Serum Cytokine Analysis. Serum was isolated from patients prior to onset of conditioning, and on days 7, 14 and 28 post-transplantation. IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, and IL-17A levels were measured using the BD Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences), according to the manufacturer’s instructions. Samples were acquired on a BD FACS Canto II flow cytometer (BD Biosciences) and analyzed using the FCAP Array v3.0.1 software (BD Biosciences). Soluble IL-6R levels were measured using the Human IL-6R alpha Quantikine ELISA Kit (R and D Systems, Minneapolis, MN). This assay has been reported to detect three forms of the sIL-6R; free sIL-6R, sIL-6R in complex with IL-6, and sIL-6R in an immune complex with Tocilizumab.

Immune Reconstitution. Multi-parameter flow cytometry was utilized for the testing of T cells and NK after mononuclear cell enrichment by density gradient separation. The antibodies used included: CD3-HPC-H7, CD4-PE-Cy, CD8-APC, and CD56-PE. Regulatory T cells were detected using the BD Pharmingen (San Diego, CA) FoxP3 staining kit according to instructions, with the addition of CD127-PE-Cy7. Tregs were defined as CD3+ CD4+ CD25+ FoxP3+ and CD127−. Th17 cells were tested after activation with PMA and Ionomycin in the presence of brefeldin A, then fixed and permeabilized before intracellular antigen staining with IL-17-PE. B cell subsets were tested on whole blood samples (300 µL) that were lysed and washed twice prior to the addition of antibodies so as to remove serum Ig that can inhibit surface Ig staining. The antibodies used included: IgD-FITC, IgM-APC, CD27-APC-H7, CD38-PE-Cy7, CD21-FITC, and CD69-APC, with initial gating on CD19+ B cells. Minimally 300,000 debris-free events were collected. The following CD19+ subsets were defined: Antigen inexperienced: CD27− IgD+, Pre-Switch Memory: CD27+ IgD+, Post switch Memory: CD27+ IgD−, Plasma cells: IgM− IgD− CD27+ CD38hi; Immature/Transitional: CD21−, Activated: CD69+. All antibodies were obtained from BD Pharmingen and testing was performed on a BD Canto II cytometer within 24 hours of cell collection. Patients in hematological relapse at the time of sampling were not assessed.
**SUPPLEMENTAL TABLE 1. DEFINITION OF EVENTS AND COMPETING RISKS FOR SPECIFIC OUTCOMES**

| Outcome                        | Event(s)                                           | Competing risks       |
|--------------------------------|----------------------------------------------------|-----------------------|
| **Event-free survival**        |                                                    |                       |
| Acute GVHD-free survival       | Grade II-IV aGVHD or death                         | None                  |
| Disease-free survival          | Relapse or death                                   | None                  |
| Overall survival               | Death                                              | None                  |
| **Cumulative incidence**       |                                                    |                       |
| Treatment related mortality    | Death                                              | Relapse               |
| Relapse                        | Relapse                                            | Death w/o relapse     |
| Acute GVHD                     | Grade II-IV aGVHD                                  | Relapse or death       |
| Chronic GVHD                   | Chronic GVHD                                       | Relapse or death       |
| Neutrophil engraftment         | 1\textsuperscript{st} of 3 consecutive days with an ANC>500 | Death or relapse or death or death w/o engraftment |
| Platelet engraftment           | 1\textsuperscript{st} day of a sustained platelet count above 20,000 w/o any platelet transfusions for the preceding 7 days | Death w/o engraftment |

All outcomes used loss to follow up / end of study as censoring
SUPPLEMENTAL TABLE 2. PATIENT CHARACTERISTICS OF CYTOKINE CONTROL POPULATION

| Variable                        | Value          |
|---------------------------------|----------------|
| N                               | 11             |
| Age, median (range)             | 61 (27-67)     |
| Sex (M/F)                       | 7/4            |
| Disease (n, %)                  |                |
| AML                             | 2 (18)         |
| MDS                             | 3 (27)         |
| Myelofibrosis                   | 2 (18)         |
| Myeloproliferative Disorder     | 1 (9)          |
| Hodgkin Lymphoma                | 2 (18)         |
| Non-Hodgkin Lymphoma            | 1 (9)          |
| Donor Type (n, %)               |                |
| MRD                             | 9 (82)         |
| MUD                             | 2 (18)         |
| Preparative Regimen (n, %)      |                |
| Myeloablative                   | 6 (55)         |
| Reduced Intensity               | 5 (45)         |
| Graft Source (n, %)             |                |
| Bone Marrow                     | 0 (0)          |
| Peripheral Blood                | 11 (100)       |

AML, acute myelogenous leukemia; MDS, myelodysplasia; MRD, matched related donor; MUD, matched unrelated donor
Supplemental Figure 1: **Acute GVHD outcomes by tissue site.** Cumulative incidence of grades II-IV acute GVHD in skin (panel A), liver (panel B), upper GI tract (panel C) and lower GI tract (panel D) of patients that received Tocilizumab for GVHD prophylaxis.

Supplemental Figure 2: **Effect of Tocilizumab administration on interleukin 6 and soluble interleukin 6 receptor levels based on conditioning regimen.** (A). Concentration of IL-6 in the serum on days 7, 14 and 28 from patients that were treated with Tocilizumab and received reduced intensity versus myeloablative conditioning. (B). Concentration of soluble IL-6 receptor in the serum on days 7, 14, and 28 from patients that were treated with Tocilizumab and received reduced intensity versus myeloablative conditioning. Statistics: *p<0.05, **p<0.01, ***p<0.001

Supplemental Figure 3: **Effect of Tocilizumab administration on cytokine production.** Concentration of IL-2, IL-4, IL-10, TNF-α, IFN-γ, and IL-17 in the serum of patients that were treated with Tocilizumab for the prevention of acute GVHD prior to the start of conditioning and at days 7, 14 and 28. Statistics: **p<0.01, ***p<0.001.

Supplemental Figure 4: **Reconstitution of Treg and Th17 subsets.** The percentage of the indicated subset within CD4+ T cells (upper panels) and the number of cells per mm3 (microliter) of blood for regulatory T cells (Tregs) and T_{H}17 cells. Data are shown for individual patients together with the median and 25th and 75th quartiles. The shaded area indicates the range of values for healthy controls. Samples were obtained at 1 month (n=33), 3 months (n=29), 6 months (n=22), and 12 months (n=13). The indicated subsets were defined as described in methods.
Supplemental Figure 5: B cell reconstitution in patients that received Tocilizumab for GVHD prophylaxis. (A,B). The number of cells per mm$^3$ (i.e., microliter) is shown in panel A, and the percentage of gated CD19$^+$ B cells is shown in panel B. The B cell subsets were defined as shown in Methods. Data are shown for individual patients together with the median and 25th and 75th quartiles. The shaded area indicates the range of values for healthy controls. Samples were obtained at 1 month (n=33), 3 months (n=29), 6 months (n=22), and 12 months (n=13).
