Azacytidine prevents experimental sclerodermic chronic graft-versus-host disease

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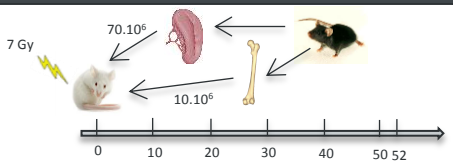
INTRODUCTION

Graft-versus-host disease (GVHD) remains one major complication of allogeneic hematopoietic stem cell transplantation (HSCT). Following unmanipulated peripheral-blood stem cell transplantation, 60% of the patients experience chronic GVHD while approximately 15% of them develop a sclerodermic form of chronic GVHD characterized by multiple organ fibrosis and loss of skin elasticity. Regulatory T cells (Treg) play a pivotal role in the pathology of chronic GVHD by inhibiting alloreactive conventional T cells. Several studies have shown that the hypomethylating agent Azacytidine (Aza) can demethylate the master transcription factor of Treg (Forkhead box protein 3, FoxP3), thus promoting Treg differentiation of conventional T cells. This work investigates the impact of Aza in a classical murine model of sclerodermic chronic GVHD (B10.D2 → BALB/cJ).

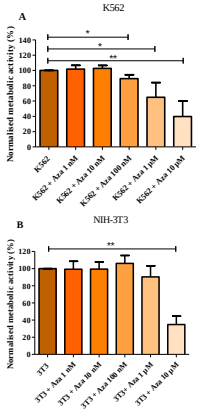
METHODS

Lethally irradiated BALB/cJ recipient mice were injected intravenously with 10.10^6 bone marrow cells + 70.10^6 splenocytes from B10.D2 donor mice. Recipients were treated with subcutaneous injections of Aza at the dose of 0,5 or 2 mg/kg every two days from day 10 to 30 following transplantation. Mice GVHD severity was evaluated for five criteria (weight loss, activity, fibrosis, hair loss and mice posture ; 0-1-2 points/criteria). Mice were sacrificed at a score of 8/10 (or > 20% weight loss) according to the ethical committee of the University of Liège.



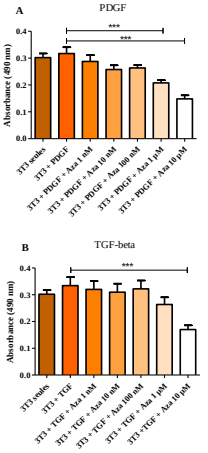
RESULTS

1. *In vitro* metabolic activity assay



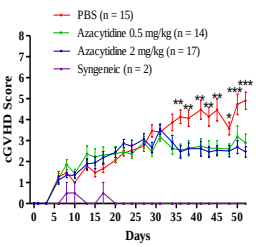
MTT assays have been performed on two different cell lines to assess the impact of Aza on metabolic activity of fibrosblasts. Aza was added to each wells at different concentration. As shown by Figure A, metabolic activity of K562 cells (used as control) decrease when Aza concentrations increase (100 nM, p=0,021 ; 1 µM, p=0,034 ; 10 µM, p=0,0068). For NIH-3T3 fibroblastic cell lines (Figure B), no effects of Aza on metabolic activity have been observed, except at higher concentration of Aza (10 µM, p=0,0003).

2. *In vitro* collagen assay



Collagen assays have been performed on NIH-3T3 fibroblastic cell lines. The amount of collagen was quantified by absorbance at 490 nm after staining of collagen fibers by Sirius Red. 500.000 cells/well were plated in triplicate and then put with various concentration of Aza and stimulated by TGF-β or PDGF. As shown by Figure A and B, the amount of collagen slightly decrease when aza concentration increase even it is not statistically significant except at higher concentration of Aza (Figure A : 100 nM, p=0,05 ; 1 µm, p=0,0005 ; 10 µM, p< 0,0001. Figure B : 10 µM, p=0,0003)

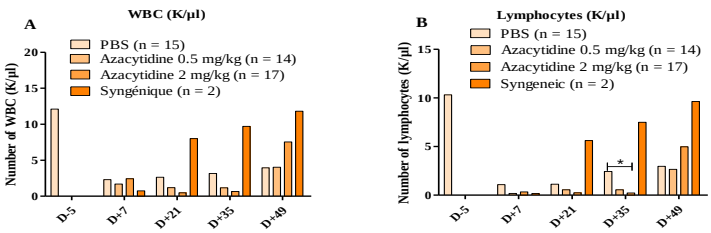
3. Mice experiment



cGVHD score following HSCT. After treatment, Aza treated mice reach a lower score compared to PBS mice (p≤0,04). Following day 35, cGVHD score of PBS mice continously increase while aza treated mice stagnate around 2-3.

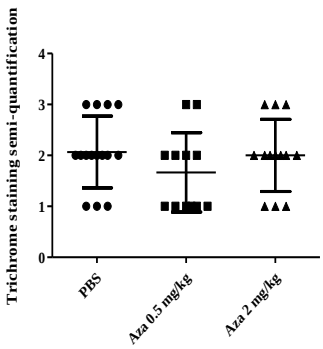
Representative pictures of mice at day 45 following HSCT

4. Cell-dyn analysis



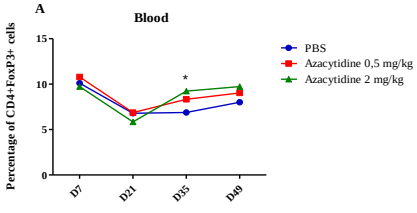
Evolution of the absolute number of white blood cells (Figure A) and lymphocytes (Figure B) after HSCT. As suggested by Figure A, the absolute number of WBC is not significantly different between the different groups of mice even if we could observe a decreased number of WBC for Aza treated mice during treatment. In contrast, the number of lymphocytes (Figure B) is significantly lower at day 35 for Aza 2 mg/kg treated mice compared to PBS mice (p=0,0479). After the end of the treatment, Aza 2 mg/kg mice rapidly recover a normal number of lymphocytes.

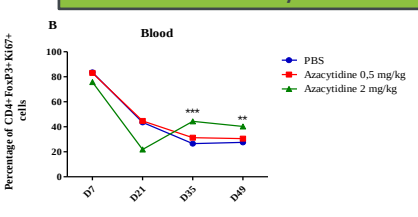
5. Collagen staining on skin biopsy at day 29

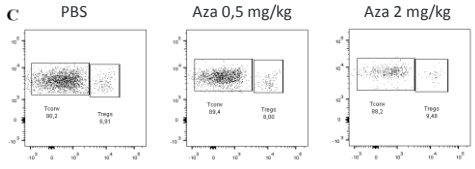


At day 29, skin biopsy were performed on mice to quantify fibrosis. After biopsy, skins sections were parrafin-embedded and collagen fibers where then stained by Massons's Trichrome. Fibrosis was blind semi-quantified for each skin slide with a score from 0 to 3 according to the area and intensity stained by Masson's trichrome. As demonstrated by the figure on the left, no statistical differences were observed between groups even if we could see a slight decrease for Aza treated mice compared to PBS mice.

6. FACS analysis







FACS analysis performed on mice blood at day 35 following HSCT. As suggested by Figure A, Azacytidine treated mice have a higher percentage of Tregs cells at day 35 (just after the end of the treatment) compared to PBS mice (p=0,03). Figure B shows that these cells are more proliferative for Aza 2 mg/kg treated mice as we could observe a significant increase of the proportion of Tregs Ki-67 positive compared to PBS mice (D35, p=0,005 ; D49, p=0,0033). Figure C shows a representative dot plot of FACS analysis on blood at day 35 for each group.

CONCLUSION

In conclusion, Azacytidine seems to be a promising treatement as it prevents cGVHD in the classical murine model of sclerodermic cGVHD. Results suggest that Aza acts mainly through immunomodulation by diminishing the number of alloreactive T cells and promoting phenotypic switch of Tconv into Tregs by demethylation of Tregs master transcription factor FoxP3.

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