Roles of metabolite polymers regulating immune responses in Rheumatoid Arthritis

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Abstract

Methylmalonic acid (MMA) is a metabolite in citric acid cycle which belongs to the group of dicarboxylic acids and is a by-product of the propionate metabolism pathway in cells. This molecule is well known to regulate CD8+ T cell responses and controls immunometabolism. Additionally, it also enhances immunosuppression. This work describes the importance of synthesized novel polymer of MMA which helps in sustained release of MMA inside the host to generate long lasting and robust immunosuppression. The effect of these polymers is first observed in peritoneal macrophages where it reduces CD80 and CD86 expression with LPS treatment. Additionally, it also reduces the in-vitro activation of T cells causing T cell anergy. We confirmed that the immunosuppressive properties of MMA polymer is not due to toxicity and increased cell death. Finally, we confirmed the in-vivo effects of the polymer where it reduced disease scores in Collagen Induced Arthritis (CIA) in DBA/1j mice where it reduces bone loss and lowers inflammation. This study is the one of the first evidence of using a polymer of MMA as a potential therapy for Rheumatoid Arthritis.

Introduction

Methylmalonic acid being a part of the propionate metabolism pathway has been shown to have immunosuppressive role specially in the context of cancer[1]. Literature suggest that MMA has potential immunomodulatory roles when looking at CD8+ T cell responses[2]. However, whether this small molecule affects overall T cell activation by altering the regulation of signaling pathways is not well understood. Additionally, the effect of MMA being a small molecule is questionable as it gets metabolized easily. Here we describe a polymer designed using MMA (Figure 1) to understand its immunosuppressive roles in macrophage and T cell responses and extrapolating its findings in a mouse model of rheumatoid arthritis.

Results

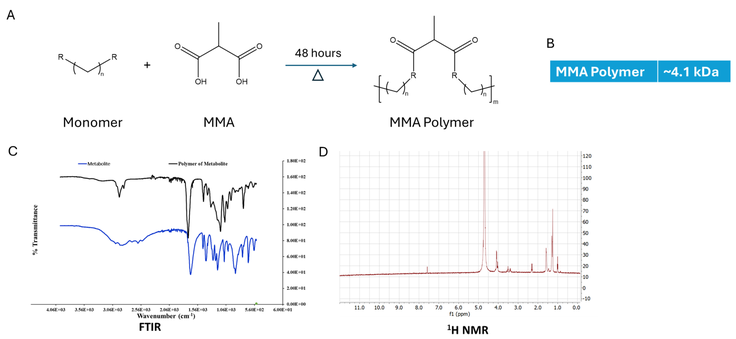

Fig. 1. MMA polymer synthesis and characterization. (A) MMA polymer synthesized by stirring the monomers for 48 hours at 70 ^{0}C. (B) The gel permeation chromatography study used for characterizing the number average molecular weight of the polymer. (C and D) Fourier Transform Infrared (FTIR) spectroscopy and proton NMR spectroscopy studies also confirms the reaction proceeded and formation of new bonds.

Methylmalonic acid and the monomer was used for generating a polymer by stirring the mixture for 48 hours at 70 ^{0}C. After the reaction time, the reaction was quenched by using ice and the polymer purified by washing with solvent and centrifugation for 5 minutes (x 3 times), Fig 1A. Synthesized polymer used for chemical characterizations. Gel permeation chromatography confirmed the formation of polymer. The number average molecular weight of polymer shown 4.1 kDa by using tetrahydrofuran (THF) as a solvent. The FTIR spectroscopy of polymer reveals the shifting of peaks to left at 1500 cm^{-1} and the formation of new narrow peak at 2900 cm^{-1} instead of broad peak as in monomer (Showing only one monomer's data, due to confidentiality). The proton NMR spectroscopy of the polymer resulted new peaks at 4 ppm and 2.2 ppm which confirms the polymeric product formation and supports the GPC and FTIR analysis.

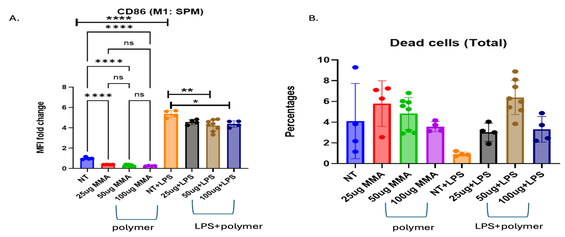

Fig. 2. A. Polymer of MMA inhibits M1 macrophage responses by reducing CD86 **B.** MMA polymer increase cell death only in LPS treated M1 macrophages.

C57BL/6 mice were treated with 25, 50 and 100 ug of MMA polymer via the intraperitoneal route. Peritoneal macrophages were isolated 24 hours post treatment. Cells were seeded at a density of 100,000 cells/well and treated wit 100ng/ml of LPS. Flow analysis was done 24 hours post LPS treatment. MMA polymer treatment causes marked reduction of CD86 in M1 peritoneal macrophages. To further understand whether the effect was due to the polymer's toxic effect, Live dead dye was used to analyse cell death. Figure 2B suggests that the cell death only increases in the presence of LPS treatment. Therefore, the increase in cell death is due to the immunosuppressive properties of the polymer

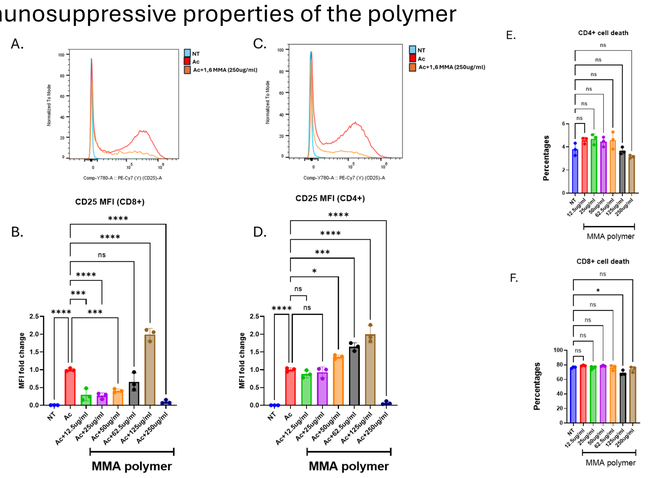

Fig. 3. MMA polymer causes reduction in CD25 expression on T cells with activation without increasing non-specific cell death. (**A and B**) Histogram and bar graphs showing CD25 MFI with increasing doses of MMA polymer in activated CD8+ T cells(**C and D**) Histogram and bar graphs showing CD25 MFI with increasing doses of MMA polymer in activated CD4+ T cells. (**E and F**) Bar graphs depicting cell death in un-activated T cells exposed to increasing doses of MMA polymer

CD3 T cell isolation was performed using splenocytes. Cells were then activated with plate bound anti-CD3 and soluble anti-CD28 in the presence or absence of increasing doses of MMA polymer. 24 hours post activation flow analysis was done to understand the expression of CD25 on T cells which showed a bimodal regulation with the polymer. Noticeably 250ug/ml of the MMA polymer showed a dramatic drop in CD25 MFI expression (Figure A-D). We further confirmed that the drop in CD25 MFI was not due to its toxic effect on cells from Figure E and F which showed no increase in cell death in un-activated T cells treated with MMA polymer.

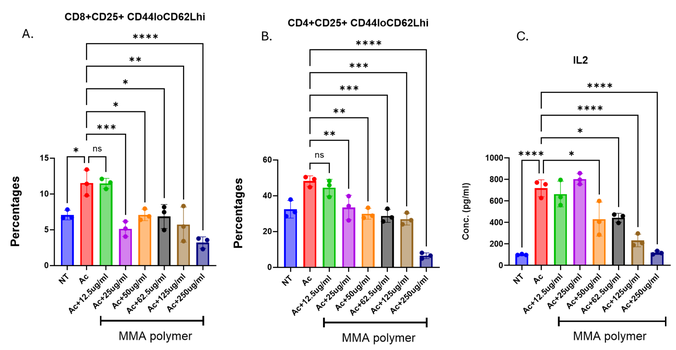

Fig. 4. MMA polymer causes reduction of CD25+CD44loCD62Lhi T cells by an IL2 dependent mechanism. (**A and B**) Bar graphs showing percentages of CD25+ "naïve" like T cells with increasing doses of MMA polymer. (**C**) IL2 levels estimated from cell supernatant of activated cells treated with increasing doses of MMA polymer

Flow analysis was done on the high CD25 expressing T cells where it was observed that the relative frequency of CD44loCD62L high T cells significantly reduced with MMA polymer treatment. To understand the initial reason for this change, IL2 ELISA was performed where a similar trend was observed suggesting the MMA polymer was inhibiting T cell responses by an IL2 dependent mechanism.

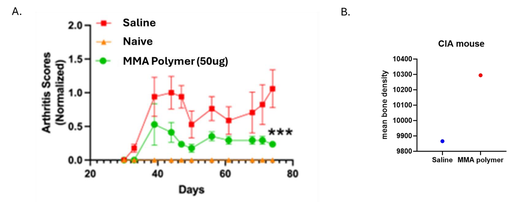

Fig. 5. MMA polymer resolves disease symptoms and reduces bone loss in CIA model of mice. (**A**) Normalized Disease scores plotted for Naive, CIA controls (Saline) and MMA polymer treated mice (**B**) micro CT analysis done on paws to calculate mean bone density loss between CIA control (saline) and polymer treated mice

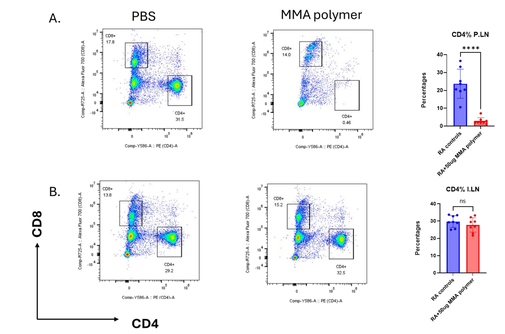

Fig. 6 . MMA polymer treatment causes loss of CD4+ T cells in draining lymph nodes of CIA model of mice. Flow plots and bar graphs depicting percentages of CD4+ and CD8+ compartments of (**A**) Popliteal lymph nodes (P.LN) and (**B**) Inguinal lymph nodes (I.LN).

DBA/1j were injected via subcutaneous route near the base of the tail with first an emulsion of bovine collagen II (bcII) antigen and CFA. 21 days post treatment, mice were given an injection of bcII and IFA via the same route. Disease symptoms were accelerated using LPS injected a week after the second injection via the intraperitoneal route. MMA polymer treatment was given to the group after LPS injections. Disease scores were calculated in regular intervals.

Figure **5 A and B** suggests that MMA polymer treatment causes marked reduction in disease scores and resolving paw inflammation. It also enhanced protection against bone loss via the micro CT studies done on mice paws at day 74. Flow analysis suggest that there is a specific and considerable loss of activated CD4+ T cells in P.LN. which is the draining lymph node in CIA mice which confirms the immunological role of this polymer in reducing inflammation **(Figure 6).**

Discussion

Polymers made out of MMA suggests to show a holistic immunosuppressive effect on both macrophages and T cells. In T cells it causes reduction of CD25 expression and lowers the percentage of activated cells which eventually get converted to memory T cells. This polymer does it via an IL2 dependent mechanism. Interestingly the polymer does not have toxic effects even at high doses of 250ug/ml. Extrapolating findings in a CIA model suggest the polymer treatment reduces disease scores, protects against bone loss and causes reduction of activated CD4+ T cells in the draining lymph nodes. This shows the potential therapeutic effects of metabolite based polymer in autoimmunity.

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