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Bifidobacterium animalis subsp. *lactis* 420 mitigates the pathological impact of myocardial infarction in the mouse

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Abstract

There is a growing appreciation that our microbial environment in the gut plays a critical role in the maintenance of health and the pathogenesis of disease. Probiotic, beneficial gut microbes, administration can directly attenuate cardiac injury and post-myocardial infarction (MI) remodelling, yet the mechanisms of cardioprotection are unknown. We hypothesised that administration of *Bifidobacterium animalis* subsp. *lactis* 420 (B420), a probiotic with known anti-inflammatory properties, to mice will mitigate the pathological impact of MI, and that anti-inflammatory T regulatory (T_{reg}) immune cells are necessary to impart protection against MI as a result of B420 administration. Wild-type male mice were administered B420, saline or *Lactobacillus salivarius* 33 (Ls-33) by gavage daily for 14 or 35 days, and underwent ischemia/reperfusion (I/R). Pretreatment with B420 for 10 or 28 days attenuated cardiac injury from I/R and reduced levels of inflammatory markers. Depletion of T_{reg} cells by administration of anti-CD25 monoclonal antibodies eliminated B420-mediated cardio-protection. Further cytokine analysis revealed a shift from a pro-inflammatory to an anti-inflammatory environment in the probiotic treated post-MI hearts compared to controls. To summarise, B420 administration mitigates the pathological impact of MI. Next, we show that T_{reg} immune cells are necessary to mediate B420-mediated protection against MI. Finally, we identify putative cellular, epigenetic and/or post-translational mechanisms of B420-mediated protection against MI.

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Supplementary material

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Keywords

cardiovascular disease; inflammation; probiotics; regulatory T cells; *B. animalis* ssp. *lactis*

1. Introduction

The bidirectional relationship between commensal gut microbiota and the intestinal immune system regulates the balance of both innate and adaptive immune responses in health and disease (Hakansson and Molin, 2011). By FAO/WHO definition, probiotics are viable microorganisms that confer health benefits to the host once consumed in adequate amounts (FAO/WHO, 2001). Because of their direct contact with the gastrointestinal tract, and given that the main site of immune activity resides in the intestine (Brandtzaeg, 2009), it is apparent why probiotics are predominantly known for their benefits to gut and immune health. However, an emerging paradigm outlines a causal role for harmful gut microbiota and translocation of bacterial components in the development of metabolic disorders, such as diabetes, obesity and cardiovascular disease (CVD) through exacerbation of pro-inflammatory mediators (Cani *et al.*, 2007a,b; De La Serre *et al.*, 2010). Consequently, addressing inflammation is now recognised as tantamount to the treatment of these disorders.

Although many studies in animals and humans have shown that probiotics and probiotic dairy products may protect against CVD, this benefit is typically mediated by lowering CVD risk factors like hypertension or hypercholesterolemia (Chen and Konhilas, 2013; Ebel *et al.*, 2014). Recent work by Hazen and colleagues outlines a congruous story that gut microbiota are active participants in the development of atherosclerosis providing a key mechanistic link between the gut, diet and CVD (Wang *et al.*, 2011). Similarly, in a series of recent experiments, oral administration of vancomycin, a broad-range antibiotic, attenuates cardiac ischemia injury presumably by disruption of gut microbiota (Lam *et al.*, 2012). Further in this study, feeding rats a commercially available probiotic (*Lactobacillus plantarum* 299v and *Bifidobacterium lactis* (Bi-07)) decreases infarct size and improves post-myocardial infarction (MI) cardiac function. Similarly, using a rat model of MI, the probiotic *Lactobacillus rhamnosus* GR-1 protects against cardiac injury (Gan *et al.*, 2014). In these studies, the investigators attribute the protection to an increase in serum leptin levels without additional mechanistic insight.

Cardiac remodelling following MI is initiated by a robust and coordinated inflammatory charge characterised by chemical signals and recruitment of distinct populations of inflammatory cells including monocytes that differentiate into macrophages. This coordinated response is perturbed *a priori* under heightened inflammatory conditions (Nahrendorf *et al.*, 2007). Consequently, any conditions that elevate peripheral or local inflammation such as high fat diet, hypertension, obesity, diabetes, periodontal disease and now harmful gut microbiota (Cani *et al.*, 2007b; Gomez-Hurtado *et al.*, 2011; Hakansson and Molin, 2011) have the potential to aggravate acute events leading to myocardial damage (Slepian and Gottehrer, 2009; Zuidema and Zhang, 2010). It is now appreciated that probiotics can suppress peripheral inflammation and that this is due to suppression of pro-

inflammatory pathways (Toral *et al.*, 2014) and, concurrently, induction of anti-inflammatory pathways (Mengheri, 2008; Smelt *et al.*, 2013).

In this series of experiments, we hypothesised that administration of *Bifidobacterium animalis* subsp. *lactis* 420 (B420) or *Lactobacillus salivarius*-33 (Ls-33) to mice will mitigate the pathological impact of MI, and that anti-inflammatory T regulatory (T_{reg}) immune cells are necessary to impart protection against cardiac injury as a result of B420 or Ls-33 administration. B420 administration has improved metabolic health and reduced markers of tissue inflammation (Amar *et al.*, 2011; Stenman *et al.*, 2014) and Ls-33 is a commercially available strain in wide use known to be beneficial for intestinal and immune disorders (Fedorak, 2008), making them good candidates for cardio-protection in our study. Towards this end, we demonstrate that B420, but not Ls-33 administration attenuates MI injury in hearts of mice following an ischemia/reperfusion (I/R) protocol. We also show that modest T_{reg} depletion eliminates the cardio-protection in B420-treated mice indicating that T_{reg} cells are necessary for the cardio-protection instigated by B420 administration. Finally, we identify putative cellular, epigenetic and/or post-translational mechanisms of B420-mediated protection against MI.

2. Materials and methods

Animals, diet and probiotic administration

All experiments were performed using protocols that adhered to guidelines and approved by the Institutional Animal Care and Use Committee at the University of Arizona and to 2011 NIH guidelines for care and use of laboratory animals. At 10 weeks of age, wild-type male mice (C57Bl/6J; Jackson Laboratories, stock 000664, Bar Harbor, ME, USA) were randomised to either standard rodent chow (NIH-31: 18% fat, 59% carbohydrate, 23% protein; Research Diets, Inc., New Brunswick, NJ, USA) or high-fat diet (#D12331: 58% fat, 25.5% simple carbohydrate, 16.4% protein; Research Diets, Inc.) that was continued throughout the study duration. High fat (HF) feeding was initiated 14 days prior to initiation of gavage protocol and continued throughout the study. Within each dietary group, mice were randomised and administered (daily by gavage; 10^9 cfu/500 μl saline) *B. animalis* subsp. *lactis* 420 (B420) (DuPont Nutrition & Health, Kantvik, Finland; ATCC: SD6685), *L. salivarius* (Ls-33; a commercially available strain known to be beneficial for intestinal and immune disorders (Collado *et al.*, 2007; Daniel *et al.*, 2006; Fedorak, 2008)) (DuPont Nutrition & Health), or saline (500 μl). We implemented a long-term (28 day) or short-term (10 day) gavage protocol, which is illustrated in the supplemental data (Supplementary Figure S1).

Probiotic batch stability

Probiotic batch stability and potency can vary depending on storage length and conditions. For this reason, we have implemented a quality control in which we determine cfu for each batch of probiotic prior to the initiation of the gavage protocol. This strategy will assure standardisation for each experimental group. To do this, freeze-dried probiotic powder is rehydrated in De Man Rogosa and Sharpe (MRS) broth at room temperature. Serial dilutions are made from this rehydrated powder and then plated on MRS-agar plates in triplicate.

Plates are then incubated under anaerobic conditions at 37°C for 72 h. Plates having between 25 and 250 colonies are counted for the determination of cfu/batch.

Surgical procedures

Mice were anesthetised with an intraperitoneal injection of 250 mg/kg tribromoethanol (Sigma, St. Louis, MO, USA), intubated and ventilated with 0.5–2.0% isoflurane (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). A single injection of Buprenorphine-SR (Reckitt Benckiser Healthcare, Slough, UK) at 1 mg/kg body weight is given prior to surgery. An ischemia reperfusion protocol was utilised in this study. A left anterior thoracotomy was performed to expose the heart and the left coronary artery (LCA) visualised. The LCA was occluded using 8-0 suture compressing a small piece of tubing (PE-10) to prevent vessel damage during occlusion. Blanching of the myocardium was used to confirm ligation and assure similar infarct size. After 30 min of occlusion, the ligature was removed and the animal was allowed to recover for a 3-day reperfusion period.

RT-PCR

Total RNA was isolated from the infarct area of hearts using the RNeasy Midi Kit RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total cDNA was generated using the NCode™ First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Maxima™ SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for real time PCR reactions. 18S RNA was used as an internal control for real time PCR. RT-PCR specific primer sequences are provided in Supplementary Table S1.

Determination of infarct size and immunohistochemistry

Following the 72-h reperfusion period, mice were anesthetised with an intraperitoneal injection of 250 mg/kg tribromoethanol (Sigma), the LCA was re-occluded at the same original site. Evans Blue (EB) dye (1%; Sigma) was then perfused retrograde through the aorta *in situ*, staining the whole heart except the left coronary bed and outlining the ischemic zone or area at risk (AAR). The hearts were excised, transversely sectioned at 1 mm thickness, and incubated in 1% 2,3,5-triphenyltetrazoliumchloride stain (TTC; Sigma). After incubation, infarcted myocardium stained white (area of necrosis; AON), viable tissue within AAR stained red, and perfused tissue remained blue. AAR and AON were measured for each section using image analysis software (ImageJ, https://imagej.nih.gov/ij) and infarction size was reported as a percentage of AON to AAR. The sections were then fixed in 10% formalin overnight, paraffin embedded, sectioned at 5 μm, and stained with haematoxylin and eosin (H/E) or with antibodies for immunological and inflammatory markers. The HRP-labelled secondary antibodies were visualised with diaminobenzidine (DAB) and counterstained with haematoxylin (Axio Imager M1; Zeiss, Oberkochen, Germany). The antibodies used are anti-IL-6 (abcam) anti-ICAM(CD54) (Cell Signaling Technologies, Danvers, MA, USA), and anti-IL-10 (LifeSpan BioScience, Seattle, WA, USA). Expansion and/or infiltration of immunohistological markers was determined by measuring the area of markers in each heart section and normalising to the area of the entire heart section (IHC area/total heart area). Next, normalised IHC area was correlated with AAR normalised to total heart area in the same heart section.

Isolation of splenic T_{reg}

Splenic T_{reg} cells were extracted using the EasySep Mouse CD25 Regulatory T cell Positive Selection Enrichment Kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol.

Flow cytometry

T cell populations were determined from splenic single cell suspensions. Viability of cell suspensions was determined using LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen) according to manufacturer's protocol. Non-specific binding was blocked with anti-mouse CD16/32 (eBioscience, San Diego, CA, USA) and then cells were stained with a panel of fluorescently labeled antibodies specific for detecting T_{reg} (anti-mouse CD3 (FITC; BD Biosciences, San Jose, CA, USA), anti-mouse CD4 (PE-Cy7; BD Biosciences), anti-mouse CD8 (Alexa Fluor 700; BioLegend, San Diego, CA, USA), and anti-mouse CD25 (eFluor 450; eBioscience), anti-mouse Foxp3 (Alexa Fluor 647; BD Biosciences), anti-mouse Helios (PE; BD Biosciences), and IL-10 (PE; BioLegend)) After staining, cells were resuspended in cell cytometry staining buffer and analysed by flow cytometry (BD LSRII; BD Biosciences). Results were analysed using FloJo software (FloJo, Ashland, OR, USA).

Depletion of CD25+ cells

Depletion of CD25+ T cells was achieved with an anti-CD25 monoclonal antibody (CD25-mAb; Clone PC61, (BioXCell, West Lebanon, NH, USA)) (Setiady *et al.*, 2010). Using a single IP injection of the CD25-mAb (IgG used as a control) the morning of I/R surgery, CD25+FoxP3+ T cells (CD4+CD3+) were depleted by about 40% 1-day post injection (data not shown). At the end of the 3-day reperfusion period, we were able to achieve a ~35% depletion of CD25+, FoxP3+ T cells (CD4+, CD3+).

Western blot analysis

T_{reg} cell lysates were prepared by mechanical disruption in a protein extraction buffer (in mmol/l): Tris(hydroxymethyl)-aminomethane (50); ethylene glycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (0.5); EDTA (1); dithiothreitol (DTT) (0.5) (pH 7.0). The buffer also contained (in mmol/l) leupeptin (0.1), pepstatin (0.1), phenylmethylsulfonyl fluoride (0.1) to prevent non-specific proteolysis. Heart tissue was lysed as described in the multiplex immunoassay section. SDS-PAGE was performed followed by transfer to a membrane (polyvinylidene difluoride [PVDF]) for Western Blot analysis. Membranes were saturated in enhanced chemiluminescence substrate and exposed by Kodak X-OMAT 2000A processor (Eastman Kodak, new York, NY, USA) onto blue basic autoradiography double emulsion film. The processed films were scanned using the Epson Perfection V750 Pro scanner (Epson America, Inc., Long Beach, CA, USA) and SilverFast Ai professional scanning software (LaserSoft Imaging AG, Kiel, Germany). Protein optical densities were quantified using LabImage 1D software (Kapelan Bio-Imaging GmbH, Leipzig, Germany). In addition, prior to immunoblotting, all membranes were stained with Ponceau S acid red and quantified for total protein. Next, total protein measured by Coomassie blue and/or Ponceau S was compared to total protein expression for equal loading. All immunoblot analysis was performed from the semi-quantitation of individual blots and was not compared

across blots. All immunoblot images of a given target were taken from the same blot. Antibodies used are anti-histone H3 (Cell Signaling Technologies), anti-acetylated histone H3 (Cell Signaling Technologies), anti-CD206 (R&D Systems, Inc., Minneapolis, MN, USA).

Multiplex immunoassay

Infarct tissue was rapidly excised 24 and 72 h post I/R and snap frozen in liquid nitrogen. Infarct tissue was then immersed in lysis buffer (Sigma CelLytic MT Mammalian Tissue Lysis Reagent containing Sigma protease inhibitor cocktail and Sigma phosphatase inhibitor cocktail 2) homogenised using a Bullet Blender® (NextAdvance, Averill Park, NY, USA). Mouse multiplex kits (Merck Millipore, Darmstadt, Germany) were used to determine cytokine and chemokine levels according to the manufacturer's recommendations.

Data and statistical analysis

Results are presented as mean ± standard error of the mean. The differences between infarct size between mice eating a HF and normal fat (NF) administered B420, Ls-33 or saline were analysed with a two-way ANOVA followed by a Tukey's HSD post hoc test. The differences between experimental groups were analysed with a Student's t-test, one-way ANOVA followed by a Tukey's HSD post hoc test, or two-way ANOVA followed by a Bonferroni post-hoc test; $P<0.05$ was considered as significant.

3. Results

B420 administration reduces infarct size following I/R protocol

We predicted that probiotic administration would attenuate myocardial damage from ischemia/reperfusion (I/R) injury. Previous work demonstrated that 4–6 weeks of B420 administration reduces inflammatory markers and weight gain related to high-fat feeding (Amar *et al.*, 2011; Stenman *et al.*, 2014). Accordingly, we subjected male mice (10 weeks of age) to 28 days gavage with B420, Ls-33 or saline. Two weeks prior to the initiation of gavage, the HF group was started on the HF diet that was continued throughout the entire study protocol. After 28 days of gavage, mice from the experimental groups described above were subjected to our I/R protocol (Lefer and Bolli, 2011). Myocardial damage was quantified by comparing the AON to the AAR. Percent infarct size is defined as AON (white) relative to the AAR (pink). On average, HF and NF mice treated with B420 displayed a significant ($P<0.05$) reduction in infarct area when compared to saline- and Ls-33 treated mice (Figure 1; Supplementary Figure S2).

B420 administration attenuates inflammatory transcriptional profile in infarct area

Since cardiac injury after ischemia/reperfusion results in an inflammatory response (Marchant *et al.*, 2012), we wanted to perform a global assessment of inflammation in the infarct area following MI. It is now appreciated that probiotics can suppress peripheral inflammation (Mengheri, 2008) and increase activity of CD4+, CD25+ and FoxP3+ T_{reg} cells (Smelt *et al.*, 2013). Reductions in the T_{reg} population is evident in patients with congestive heart failure (CHF) and lower levels of T_{reg} associate with worse prognosis in CHF patients with reduced cardiac function (Okamoto *et al.*, 2014). Using RT-PCR, we

determined gene expression for tumour necrosis factor alpha (TNF-α), interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1), forkhead-winged helix P3 transcription factor (FoxP3) and IL-17 in the infarct and peri-infarct area encompassing the border zone region of infarcted hearts.

Sham-operated controls showed minimal (3–6 fold lower expression than post-MI hearts), if any, expression of pro-inflammatory cytokines and were omitted from the bar graph for clarity. As illustrated in Supplementary Figure S3A, the border zone myocardium from HF mice demonstrated a reduction in pro-inflammatory indicators, TNF-α, IL-6, and MCP-1 in B420-treated compared to saline-and Ls33-treated hearts. Although IL-17 trended towards a reduction in B420-treated hearts, this was not statistically significant. Border zone myocardium from NF mice demonstrated a slightly different pattern of cytokine activation with minimal, if any, changes in these same markers (Supplementary Figure S3B). Of particular significance, hearts from B420-administered mice fed either a HF or NF demonstrated an increase in FoxP3 transcriptional expression, suggesting an increase in the T_{reg} population of cells. Incidentally, B420 or Ls33 administration in sham-operated mice did not significantly alter inflammatory markers when compared to sham-operated controls (data not shown).

B420 administration minimises pro-inflammatory cytokines in infarct area

Measures of mRNA transcripts as above only predicts protein expression (Guo *et al.*, 2008). Moreover, therapeutic efficacy is typically evaluated by infarct size and histological assessments (Takagawa *et al.*, 2007). To determine the localisation of the inflammatory response, immunohistochemistry was performed on heart sections for the presence of the inflammatory markers: IL-6 and intercellular adhesion molecule-1 (ICAM-1). H&E staining of infarcted hearts confirmed infarct location and size and matched determinations of EB and TTC staining. In each of the images, IL-6 and ICAM-1 staining paralleled infarct size demonstrating that B420, but not Ls-33 can suppress the absolute amount of IL-6 secretion and subsequent ICAM-1 expression (Figure 2; Supplementary Figure S4).

In order to better quantify IL-6 and ICAM-1 staining, the area of cytokine infiltration was normalised to the total cross-sectional area of each heart section. Next, the normalised cytokine area was plotted against the AON in the identical section, which was also normalised to total cross-sectional area of each heart section. Linear regression analysis represents the ‘correlation’ between infract size as AON to cytokine infiltration. The line of identity indicates a 1:1 correlation between cytokine and AON and is illustrated as the grey line in Supplementary Figure S4. As shown in Supplementary Figure S3, the area of coverage for IL-6 (1.11±0.09; r2=0.918, $P<0.0001$) more closely matched the AON than the area of coverage for ICAM-1 (0.56±0.15; r2=0.538, $P=0.0028$).

B420 administration for 7–10 days reduces infarct size following I/R protocol

In order to elevate the clinical and translational potential of these studies, we wished to test a shorter administration period. Since we did not see an effect of Ls-33 on infarct size, we did not include this group in the following studies. We subjected another group of mice to HF feeding and B420 gavage followed by I/R at 7 days from the start of gavage. Following the

3-day reperfusion period brining to the total number of B420 administration to 10 days, hearts were excised and analysed for infarct size (Figure 3). Again, B420-treated mice demonstrated a significant reduction in infarct area when compared to saline-treated animals (Supplementary Figure S5).

B420 administration increases epigenetic and post-translational modifications of T_{reg} cells

The mechanism by which probiotics suppress peripheral inflammation may be due to induction of T_{reg} from dendritic cells in the gut and periphery (Mengheri, 2008). Accordingly, we measured peripheral (splenic, blood, and heart draining mediastinal lymph nodes) T_{reg} populations. Unexpectedly, 4 weeks of B420 administration did not impact T_{reg} cell numbers in the circulation, mediastinum or spleen (Supplementary Figure S6). This suggests that B420 treatment does not increase the quantity of T_{reg} cells. We also established that a HF diet does not increase T_{reg} populations compared to a NF diet (Supplementary Figure S7). Recent work shows that metabolites from the gut may act directly on the T_{reg} population through epigenetically modified conserved non-coding sequences (CNS) in the *foxp3* locus or increased acetylation of FoxP3 protein increasing the expression and functionality of T_{reg} cells *in vitro* and *in vivo* (Arpaia *et al.*, 2013; Wang *et al.*, 2009). Therefore, we tested the hypothesis that B420 modification of the gut microbiome enhanced global acetylation of T_{reg} cells. For this experiment, splenic T_{reg} were isolated and probed for acetyl-histone 3 (Ac-H3) and total histone (H3) using antibodies specific for Ac-H3 and H3. Figure 4 (and Supplementary Figure S8) illustrates that Ac-H3 normalised against H3 was elevated in B420- compared to saline-treated mice. The indication is an increase in epigenetic and post-translational remodelling in the T_{reg} population.

CD25+FoxP3+ T_{reg} cells are necessary for B420-mediated protection against cardiac injury

The increase in T_{reg} histone H3 acetylation suggests an increase in T_{reg} activity following B420 administration despite no measureable increases in T_{reg} cell numbers. The suggestion is that B420 may expand a subset of the T_{reg} population into a more functional and more immunosuppressive phenotype. Interestingly, complete depletion of the T_{reg} population exacerbates cardiac injury post-MI (Weirather *et al.*, 2014), while superagonism using a CD28 antibody ameliorates pathological remodelling post-MI (Tang *et al.*, 2012). If activated T_{reg} cells are playing a predominant role in gut-induced (following B420 administration) cardio-protection, we predicted that T_{reg} depletion would eliminate cardio-protection in B420-treated and not saline-treated mice. Accordingly, we employed a strategy to moderately deplete the T_{reg} population. We chose a moderate depletion for 2 reasons: (1) complete T_{reg} depletion may mask the protective effect of activated T_{reg} cells, and (2) recruitment of inflammatory cells, specifically T_{reg}, to the site of MI occurs within 3 days (Frangogiannis, 2012; Weirather *et al.*, 2014).

Although a number of strategies have been employed to inhibit or deplete T_{reg} cells (some of which are currently used clinically (Pere *et al.*, 2012)), we used an anti-CD25 monoclonal antibody (CD25-mAb; Clone PC61) to selectively deplete the T_{reg} population (Setiady *et al.*, 2010). Using a single intraperitoneal injection of the anti-CD25-mAb (IgG used as a control) the morning of I/R surgery, CD25+FoxP3+ T cells (CD4+CD3+) were depleted by about ~40% 1 day post injection (data not shown) and at the end of the 3-day reperfusion period,

we were able to achieve a ~50–60% depletion of CD25+FoxP3+ T cells (CD4+, CD3+) (Supplementary Figure S9). Therefore, we subjected mice to HF feeding (continued throughout the study protocol), B420 administration (2 weeks), and CD25-mAb or IgG injection prior to I/R. After the 3-day reperfusion period, hearts were excised and analysed for infarct size as before. Again, we observed a clear and significant effect of B420 administration on cardio-protection as above. In support of our prediction, moderate T_{reg} depletion eliminates the cardio-protection in B420- but not saline-treated mice (Supplementary Figure S10). H&E staining of infarcted hearts confirmed infarct location and size and matched determinations of EB and TTC staining. IL-6 and IL-10 staining patterned infarct size, however, there were no detectable differences in staining densities by visualisation (Supplementary Figure S11). These data show that T_{reg} cells are necessary for the cardio-protection instigated by the gut and B420 administration.

B420 administration promotes infarct repair and suppresses infarct expansion

We hypothesised that B420 attenuates pathological and promotes beneficial cardiac remodelling post-MI. In order to better assess the model that B420 attenuates MI expansion and promotes MI repair, we quantified (by multiplex immunoassay) several inflammatory analytes (cytokines and chemokines) in infarct tissue from B420- and saline-treated groups at 24 and 72 h post-I/R. To determine how both the time following the infarct and treatment (Tx) with saline or B420 impact analyte concentration, we performed a 2-way ANOVA followed by a post-hoc Bonferroni analysis (Supplementary Figure S12). First, both factors of time (24 vs 72 h) and Tx (saline vs B420) impacted the amount of interferon γ(IFN-γ) present in the infarct with a significant upregulation of IFN-γ 24 h post-MI in saline- compared to B420-treated mice. More importantly, there was a significant interaction between the factors of time and Tx indicating that B420 administration significantly affects IFN-γ concentration over the 24 to 72 h reperfusion period. There was a clear attenuation of IFN-γ inducible protein 10 (IP-10) in the infarct area of B420-treated mice. In addition, IP-10 showed significant differences in time, Tx and time by Tx (interaction). The chemokine, MCP-1, was impacted by Tx with a significant interaction between time and Tx. Furthermore, MCP-1 was significantly downregulated 72 h post-MI in B420-treated hearts. The patterning of the cytokine, IL-15, exhibited a significant attenuation at 24 and 72 h post-MI in B420-treated mice compared to saline-treated controls. Finally, CD206 expression (an M2 macrophage marker (Lorchner *et al.*, 2015)), as determined by Western blot analysis, showed significant differences in time, Tx and time by Tx (interaction). CD206 expression increases with reperfusion time (24 vs 72 h) in B420-treated compared to saline-treated controls (Supplementary Figure S13). These data suggest that there is an increase in M2 macrophages in the B420 treated group after 72 h of reperfusion post-MI.

4. Discussion

The gut is emerging as a key player in health and disease. Although incremental advances in MI prevention and treatment are continually evolving, we implemented a combinatorial and integrative approach that may accelerate gut and immune-based therapies for MI. We show a marked reduction in MI size following pre-treatment with the probiotic B420. Considering that the last 15–20 years of research has established a mechanistic link between

inflammation and every aspect of MI and that B420 treatment reverses CHF-induced inflammation (Amar *et al.*, 2011; Stenman *et al.*, 2014), we predicted that probiotic administration would minimise the impact of MI. In this study, we show that both 4 weeks and 7 days of B420 administration in mice mitigates infarct size from I/R when compared to the saline-treated group. Interestingly, we show that another probiotic strain, Ls-33, has no effect infarct size.

Bifidobacteria and lactobacilli are two distinct genera of bacteria belonging to different phyla (Ventura *et al.*, 2009). Although both strains are considered lactic acid producing, comparative genomics reveal no common genetic signatures and only 7 genes shared between bifidobacteria and lactobacilli (Lukjancenko *et al.*, 2012; Ventura *et al.*, 2009). The authors further suggest that metabolism of complex sugars may underlie *Bifidobacterium* functionality when compared to other probiotic species, such as *Lactobacillus* (Ventura *et al.*, 2009). Moreover, the genus of *Bifidobacterium* is relatively small and may harbour key differences among *Bifidobacterium* species that drive probiotic efficacy. A further pursuit of this difference in strain and genera efficacy was beyond the scope of this study, but is an interesting direction for further exploration.

In this study, we focused on a model of high-fat feeding (HF) for 2 reasons. First, diabetic and obese patients dominate the clinical ‘high-risk’ MI population. Despite being clinically heterogeneous, the burden of MI patients with diabetes and obesity continues to rise (Fang and Alderman, 2006). We posit that feeding mice a HF models the clinically relevant phenotype of a patient at the highest risk for MI. Second, HF feeding increases low-grade systemic inflammation (Cani *et al.*, 2007b) caused in part by the growth and translocation of harmful bacteria induced by HF feeding (Cani *et al.*, 2008), and B420 treatment reduces weight gain and inflammation in HF fed mice (Stenman *et al.*, 2014). Since B420-mediated cardio-protection occurred in mice eating the standard NF rodent chow we were confident that the HF diet does not introduce significant confounders to the mechanism of B420-mediated protection, and is the most representative of a standard western diet.

Although the cardio-protective effects of probiotics are typically attributed to lowering CVD risk factors like hypertension or hypercholesterolemia (Chen and Konhilas, 2013) and not direct protection against ischemic damage, evidence suggests a direct role for probiotic induced cardio-protection. A recent study demonstrates that altering intestinal microbiota using the antibiotic, vancomycin, diminishes ischemic damage in the rat (Dahl S) heart (Lam *et al.*, 2012). Furthermore, supplementing the rats Goodbelly probiotic juice, which contains 2 probiotic bacteria, *L. plantarum* (Lp299v) and *B. lactis* (Bi-07), daily for 14 days also protects against I/R injury. In another study, Sprague-Dawley rats administered *L. rhamnosus* GR-1 immediately following permanent ligation of the left coronary artery show attenuation of left ventricular hypertrophy and improved haemodynamic and echocardiographic parameters (Gan *et al.*, 2014). It should be noted these effects could be species-specific, since our control strain Ls-33 shows no reduction in infarct size or expression of inflammation markers.

In our study, we pre-treated mice with B420 or Ls-33 that continued throughout the duration of the study protocol. This would be equivalent to more of a preventative measure rather

than a therapeutic. To elevate the clinical potential, we initiated B420 administration at an earlier time point with similar results; B420 remained effective at reducing infarct size. While previous work (Gan *et al.*, 2014; Lam *et al.*, 2012) initiated probiotic treatment at the time of infarct, they also used a model of permanent ligation, unlike the current study. Therefore, to elucidate the impact of B420-adminstratin on long-term cardiac remodelling, we induced a moderate MI by distal (permanent) ligation of the LCA following 4 weeks of B420 administration that was continued throughout the study protocol (Supplementary Figure S14). We show attenuation of pathological cardiac remodelling and maintenance of cardiac function in B420-treated compared to saline-treated mice. Therefore, we stipulate that the benefit of B420 administration is cardio-protective based on the extent and not the origin of myocardial damage.

The 2 aforementioned studies (Gan *et al.*, 2014; Lam *et al.*, 2012) provide evidence that Goodbelly or GR-1 administration mediates, at least in part, cardio-protection by lowering levels of leptin, which can exert hypertrophic effects at high levels(Gan *et al.*, 2014; Lam *et al.*, 2012). Our hypothesis, however, focuses on the ability of probiotic administration to modulate the inflammatory response post-MI. As part of cardiac remodelling post-MI, recruitment of distinct populations of inflammatory cells includes monocytes that differentiate into macrophages (Nahrendorf *et al.*, 2007). Macrophages are a heterogeneous cell population central to the acute MI response and critical to post-MI healing (Lorchner *et al.*, 2015). In the infarcted heart, infiltrating monocytes differentiate into cytotoxic, inflammatory macrophages (M1-type) but can be converted to reparative, anti-inflammatory macrophages (M2-type) (Frangogiannis, 2012). Studies indicate an early infiltration of specific monocyte/macrophage (Ly-6C^{hi}) populations to phagocytose necrotic cells with a second wave (Ly-6C^{lo}) to promote infarct healing (Nahrendorf *et al.*, 2007). Evidence points to better clinical outcomes that correlates with the second Ly-6C^{lo} (M2-type; infarct healing) as compared to the initial Ly-6C^{hi} (M1-type; infarct expansion) population (Nahrendorf *et al.*, 2007). Accordingly, the transcriptional profile of infarcted hearts demonstrates a shift towards an anti- from a pro-inflammatory environment post-MI in B420-treated mice. Immunohistochemical staining demonstrates upregulation of IL-6 in response to myocardial injury and is associated with post-MI remodelling (Nian *et al.*, 2004). IL-6 secretion can induce ICAM-1 expression, which promotes the adherence of infiltrating neutrophils following an ischemic episode (Frangogiannis *et al.*, 2002) both of which parallel infarct size demonstrating that B420 can also suppress IL-6 secretion and subsequent ICAM-1 expression.

In addition, T cells are recruited to the site of myocardial injury by these signals where they release pro-inflammatory mediators. At the same time, the immune system produces T_{reg} that suppress the inflammatory responses following an MI (Tang *et al.*, 2012). T_{reg} cells are pleiomorphic regulators of immune homeostasis acting to maintain self-tolerance, control autoimmune deviations, and prevent runaway immunogenic responses to pathogens (Ohkura *et al.*, 2013). They are specialised T cells characterised, in part, by the expression of FoxP3 and cell surface markers, CD4 and CD25 (Sakaguchi *et al.*, 2010). Accordingly, our data indicate an increase in FoxP3 mRNA levels in infarcted regions of the heart in B420 treated animals thus suggesting an increase in T_{reg} populations. A recent study by Weirather *et al.* (2014) validates a critical role for T_{reg} cells first, by depleting the T_{reg} population, which

exacerbates cardiac inflammation and infarct size post-MI and, second, by T_{reg}-cell activation using superagonistic anti-CD28 monoclonal antibody which improves healing and survival post-MI.

For these reasons, we hypothesised that anti-inflammatory T_{reg} cells are necessary to impart protection against an MI as a result of *B420* administration. Based on Weirather *et al.* (2014), complete T_{reg} depletion would exacerbate post-MI remodelling regardless of B420-treatment. Therefore, we employed an anti-CD25-mAb to deplete the T_{reg} population by approximately 50%. This depletion strategy does not significantly worsen infarct size in control animals but eliminates the cardio-protection in B420-treated mice. Supporting our hypothesis, it was recently shown that a combination of B420 and a prebiotic, polydextrose, differentially modulated T cell populations in the small intestinal *lamina propria* in mice that were fed a HF diet (Garidou *et al.*, 2015). On a further note, the combination of B420 and prebiotic restored the expression of Reg3β which was down regulated by the HF diet. Reg3β is an important regulator of myocardial healing in the ischemic heart (Lorchner *et al.*, 2015). It is unclear, however, how this local modulation would translate into systemic or heart-targeted effects.

Building upon the model that B420 attenuates MI expansion (M1-type) and promotes MI repair (M2-type), we quantified (by multiplex immunoassay) inflammatory analytes (cytokines and chemokines) in infarct tissue from B420- and saline-treated groups 24 and 72 h post-I/R. The general pattern of analytes (IFN-γ, IP-10, MCP-1, and IL-15) measured in the infarct area supports/validates our prediction that B420 attenuates MI expansion and promotes MI repair. IFN-γ is a cytokine of Th1 cytotoxic T lymphocytes that contributes to macrophage activation by increasing phagocytosis and priming the production of pro-inflammatory cytokines including IP-10 (Bujak *et al.*, 2009). It follows, then, that there is a clear attenuation of IP-10 in the infarct area of B420-treated mice. The chemokine, MCP-1 promotes macrophage differentiation through pro-inflammatory cytokines (Kaikita *et al.*, 2004) and remains lower in B420-treated hearts compared to saline controls. We found the patterning of the cytokine, IL-15, particularly novel and relevant. Monocytes and dendritic cells produce IL-15, which facilitates the activity of cytotoxic natural killer cells, CD8+ T cells and pro-inflammatory macrophages (Huntington, 2014; Ikemizu *et al.*, 2012). Again, 24 h post-MI, B420-treatment mitigates IL-15 that remains attenuated when compared to saline-treated mice. Finally, the enhanced shift towards CD206 positive markers (M2-type) in B420- compared to saline-treated hearts post-MI supports this transition towards reparative mechanisms. The putative mechanisms by which B420 attenuates cardiac injury is outlined in Figure 5.

In conclusion, we show that a probiotic, B420, mitigates inflammation-related cardiac conditions in mouse models, namely I/R-induced cardiac injury and permanent ligation of the left coronary artery. In comparison, a Lactobacillus strain showed no effect, demonstrating that the benefit was species-specific. We also demonstrate several aspects of the mechanism, including epigenetic modifications and T_{reg} activation. It is worth commenting on the global implications of these studies in the clinical population. Although the literature cites many examples showing a clinical benefit of specific probiotic strains for human disease, we readily acknowledge that the translation of our findings to a real, clinical

benefit for cardio-protection will be challenging. In our study, we administered 10^9 cfu, which differs from human studies using between 10^6 up to 10^{10} cfu per day (Klein *et al.*, 2008; Roessler *et al.*, 2012). Although multiple factors ultimately contribute to the lack of translational clinical efficacy, the predominant conditions impacting probiotic functionality include microbial diversity of the host gut, method of probiotic delivery, and the uncontrolled dietary matrix of the human population. The suggestion is that probiotic strains dosage may need to be titrated based on disease condition. Nevertheless, the finding that probiotics may promote cardiac health with an inflammation-related mechanism provides an entirely novel treatment strategy warrants further investigation in human subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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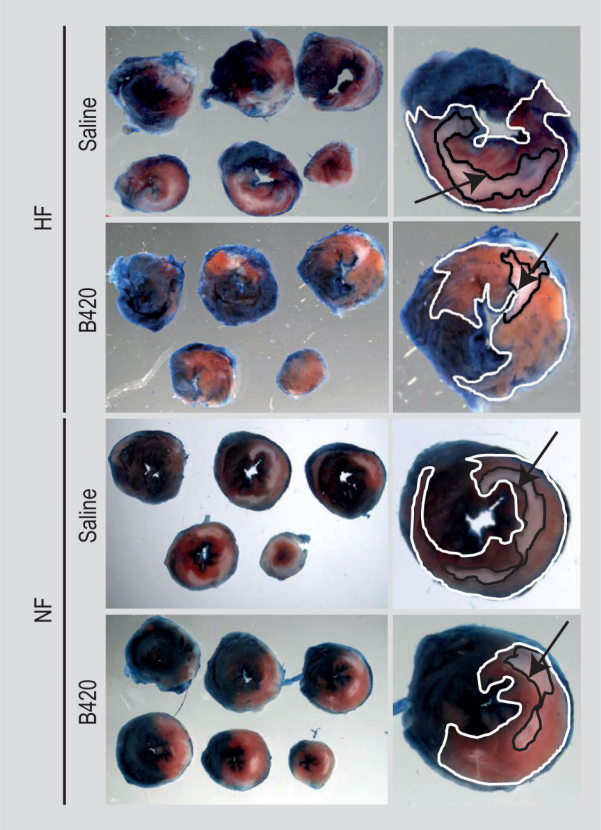

Figure 1.

Bifidobacterium animalis subsp. *lactis* 420 (B420) reduces infarct size following in ischemia/reperfusion (I/R) compared to saline, Ls-33 treated mice. Serial sections of hearts from high fat (HF) and normal fat (NF) fed mice administered saline or B420. *Lactobacillus salivarius*-33 administered hearts were omitted for clarity but matched saline controls. Enlarged section image identifying the area at risk (AAR; pink area, outlined in white) and the area of necrosis (AON; white area, outlined in black, indicated by black arrow).

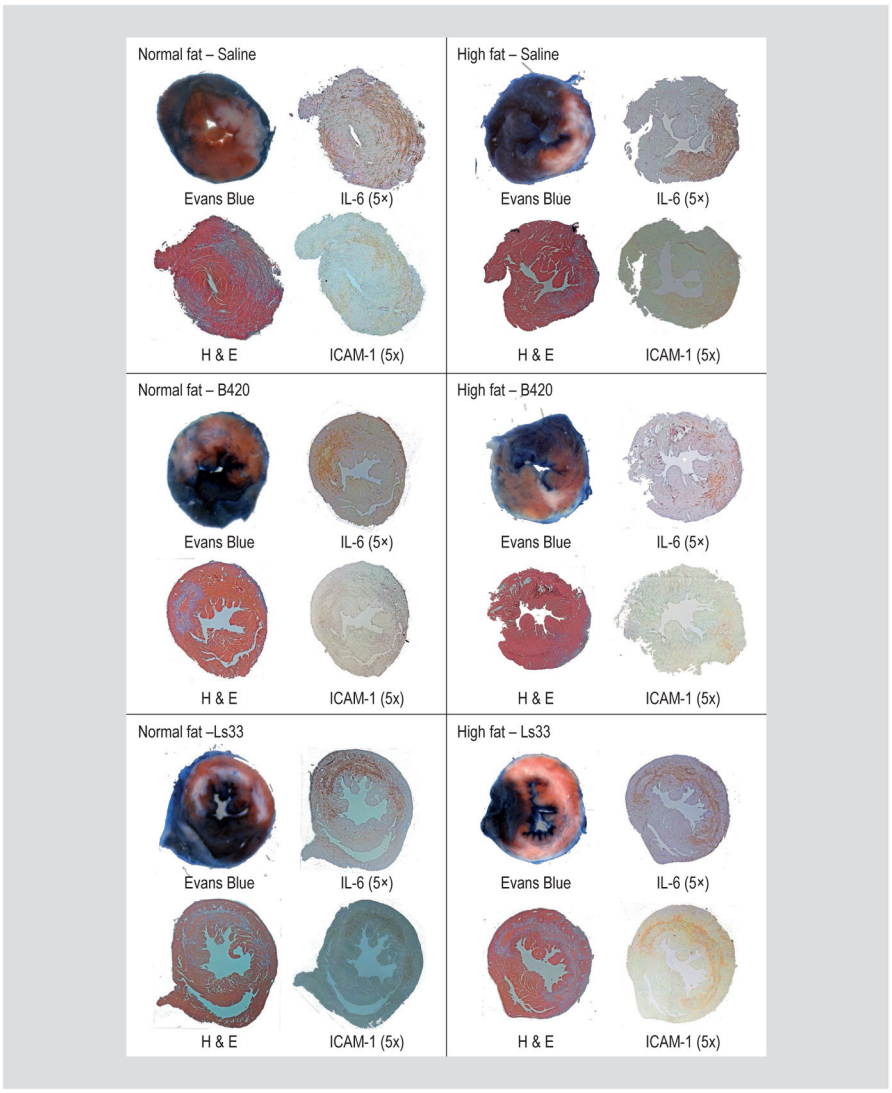

Figure 2.

Bifidobacterium animalis subsp. *lactis* 420 (B420) minimises pro-inflammatory cytokines in infarct area. Following EB and TTC staining, hearts were fixed and stained for H&E, IL-6 and ICAM-1 as indicated. In general, the extent of IL-6 and ICAM-1 expression paralleled the size of the AON and is reduced in the hearts of mice administered the B420. Hearts of normal fat and high fat fed mice administered saline (top), B420 (middle), or *Lactobacillus salivarius*-33 (Ls-33) (bottom).

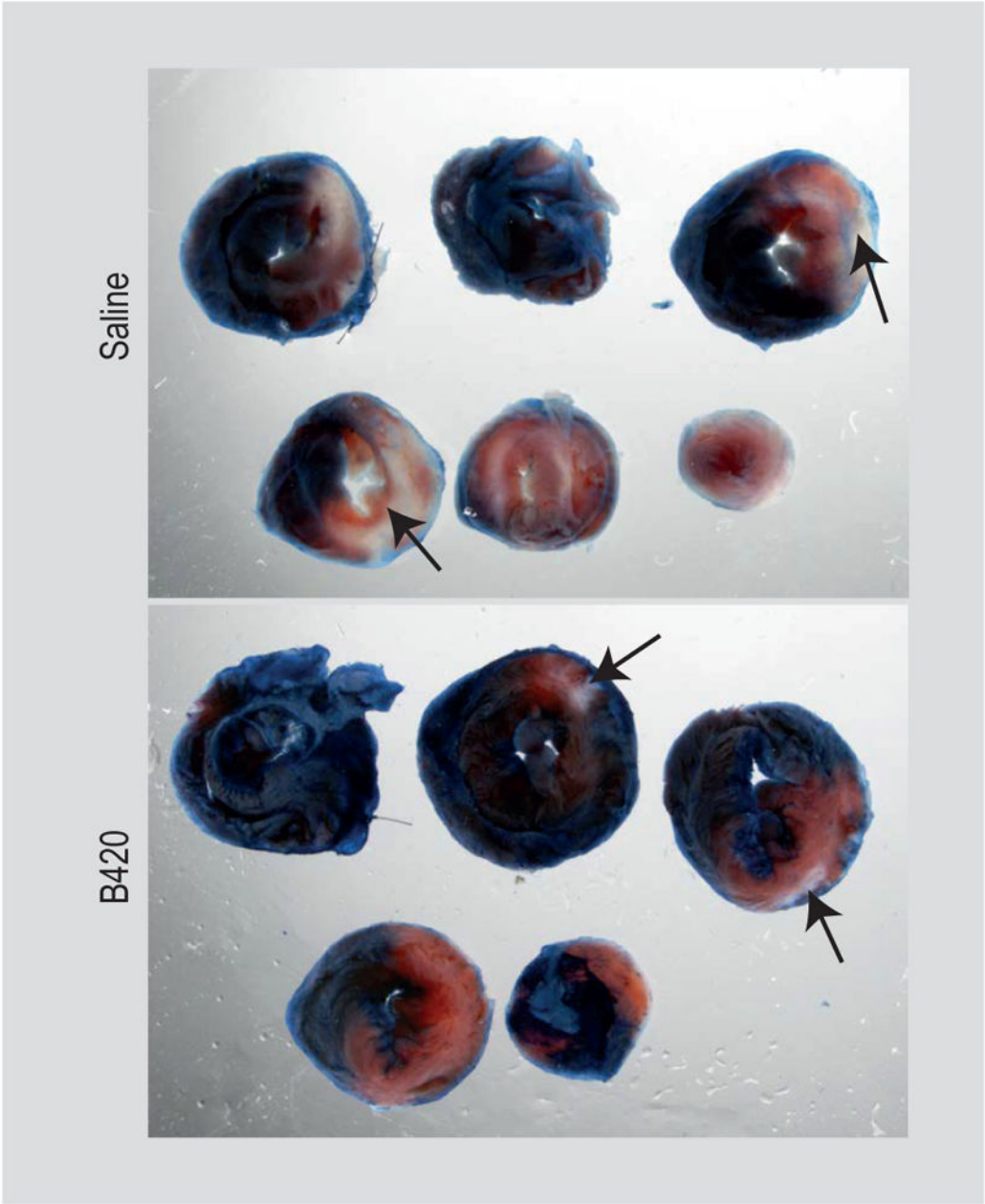

Figure 3.
Short-term *Bifidobacterium animalis* subsp. *lactis* 420 (B420) administration reduces infarct size following ischemia/ reperfusion (I/R) protocol. Serial sections of hearts from saline and B420 administered group. The area at risk (AAR) is the pink area and the area of necrosis (AON) is the white area (indicated by the black arrow).

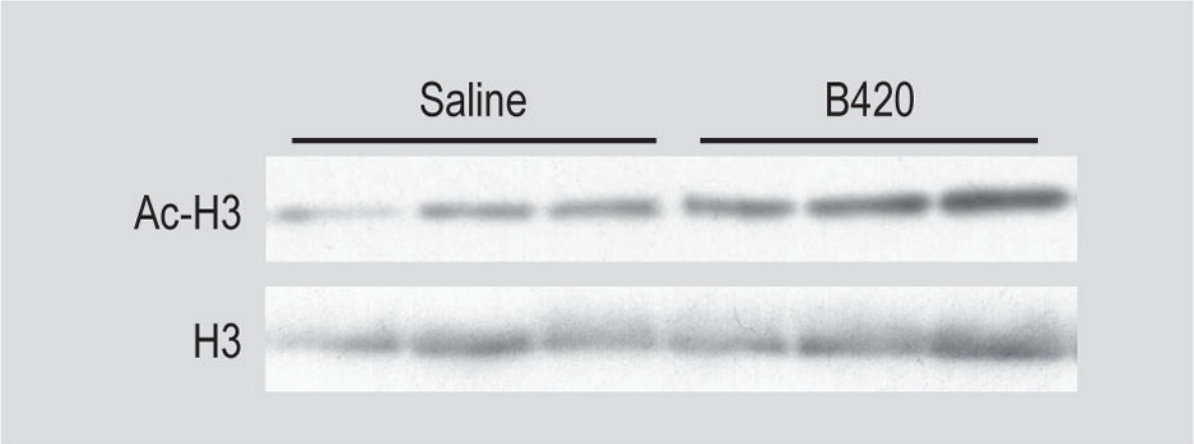

Figure 4.

Bifidobacterium animalis subsp. *lactis* 420 (B420) administration increases epigenetic and post-translational modifications in T_{reg} cells. Western blot images of acetyl-histone 3 (AC-H3) and total histone (H3) from splenic T_{reg} cells.

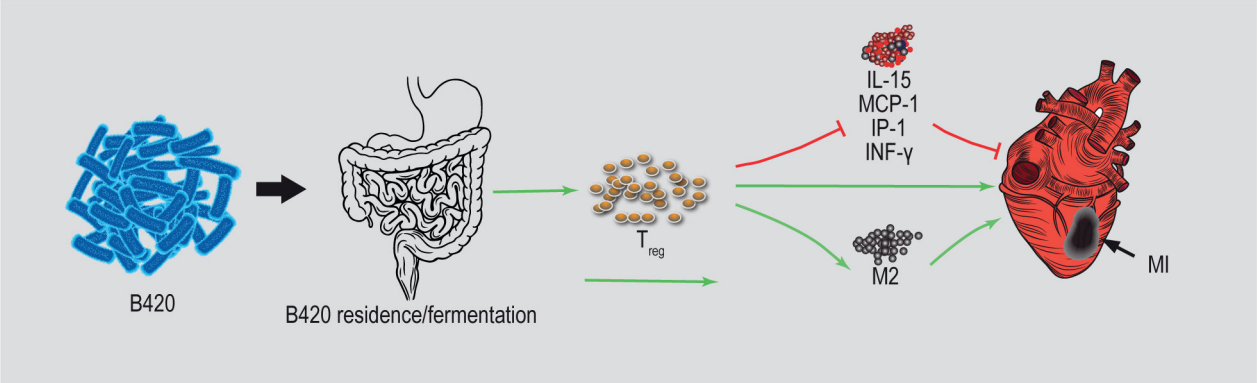

Figure 5.

Putative mechanism by which *Bifidobacterium animalis* subsp. *lactis* 420 (B420) treatment attenuates cardiac injury following myocardial infarction (MI). The putative mechanisms by which B420 may impact cardiac injury based on our data and findings in the literature. The first step is the ingestion of B420 and entry to the gut. It is unclear whether B420 colonises the gut during the administration period, but it is known that B420 fermentation and/or interaction with the gut activates a proportion of the T_{reg} population. T_{reg} activation by B420 administration either directly or through the attenuation of pro-inflammatory cytokines attenuates cardiac injury due to MI. Our data also suggests that B420 administration hastens the transition to reparative, M2 type macrophages, potentially through activated T_{reg} cells. The red arrow indicates a pro-inflammatory impact that would exacerbate MI; the green arrow represents an anti-inflammatory impact that would mitigate MI.