The Effect of Mineralocorticoid Receptor Antagonists on Recruitment and Function of Endothelial Progenitor Cells in Patients with Congestive Heart Failure

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ABSTRACT: **Background:** Circulating endothelial progenitor cells have an important role in the process of vascular repair. Impaired recruitment and function of endothelial progenitor cells is related to the pathophysiology of congestive heart failure. Endothelial progenitor cells have been shown to express the mineralocorticoid receptor.

Objectives: To investigate the effect of mineralocorticoid receptor antagonists on endothelial progenitor cells in patients with heart failure.

Methods: Twenty-four patients with compensated heart failure, who were not under mineralocorticoid receptor antagonist therapy, were recruited. Either eplerenone (n=8) or spironolactone (n=16) therapy was initiated. Circulating endothelial progenitor cell level, identified as the proportion of mononuclear cells expressing vascular endothelial growth factor receptor 2 (VEGFR-2), CD133, and CD34, was evaluated by flow cytometry at baseline and after 8 weeks. Following 7 days of culture, colonies were counted by microscopy and MTT assay was performed on randomly selected patients (n=12) to estimate viability.

Results: Both median CD34+/VEGFR2+ and median CD133+/VEGFR2+ increased significantly (P = 0.04 and 0.02, respectively). However, the number of colonies and viability of the cells after therapy (as assessed by the MTT assay) was not significantly different compared with the baseline.

Conclusions: These preliminary results suggest that mineralocorticoid receptor blockade may enhance endothelial progenitor cells recruitment in patients with compensated heart failure.

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KEY WORDS: congestive heart failure (CHF), circulating endothelial progenitor cells (EPCs), mineralocorticoid receptor (MR) antagonists

Recent evidence indicates that circulating endothelial progenitor cells (EPCs), a population of bone marrow-derived cells, have an important role in the process of vascular repair by promoting re-endothelialization following injury [1]. EPCs are primarily identified by the expression of combinations of cell-surface antigenic markers, including CD133, CD34, and vascular endothelial growth factor receptor 2 (VEGFR-2) and have the potential to differentiate into mature cells with an endothelial phenotype [2]. Impairment of EPCs is related to endothelial dysfunction [3], coronary artery disease [4], and adverse clinical outcome [5,6]. Both clinical studies and experimental models suggest that EPC impairment is strongly associated with the development and progression of congestive heart failure (CHF) [5,7]. Aldosterone, a mineralocorticoid hormone, also plays an important role in the pathophysiology of CHF. Aldosterone targets cardiomyocytes, cardiac fibroblasts, and endothelial cells, leading to cardiac hypertrophy, fibrosis, and vascular injury. High levels of aldosterone impair vascular function and predict increased risk of mortality in patients with acute myocardial infarction or CHF [8]. Mineralocorticoid receptor (MR) blockade with spironolactone or eplerenone ameliorates endothelial function in patients with CHF [9] and improves survival in patients with cardiovascular disease [10,11]. Interestingly, early EPCs were shown to express the MR and aldosterone reduced their formation in a concentration-dependent manner [12,13]. In addition, EPCs from patients with primary hyperaldosteronism displayed reduced migratory potential compared with controls of similar age. We hypothesized that MR blockade in CHF may enhance EPCs mobilization, thus having a beneficial impact on CHF pathophysiology and outcomes, independent of its well-known neurohormonal properties.

METHODS

PATIENTS AND ELIGIBILITY CRITERIA

The study included 24 ambulatory patients undergoing follow-up in the CHF clinic at Rabin Medical Center, Petah Tikva, Israel. All patients were between 20 and 80 years of age and not taking MR antagonist for at least 3 months prior to recruit-

ment. Exclusion criteria were renal insufficiency (creatinine ≥ 2.5 mg/dl), hepatic dysfunction (alanine aminotransferase ≥ 2.5 times the upper limit of normal), thrombocytopenia (< 100 × 10^3 cells/mm^3), anemia (hemoglobin < 10 g/dl), or specific contraindications for spironolactone or eplerenone. Patients with a history of hospitalization for decompensated CHF, acute coronary syndrome, or coronary revascularization in the past 3 months; malignant diseases; or hematological disorders were also excluded. The study was approved by the institutional review board and ethics committee of the Rabin Medical Center, and all subjects provided written informed consent.

MINERALOCORTICOID ANTAGONIST THERAPY

Patients were initially treated with 12.5 mg of spironolactone daily. If tolerated, dosage was increased to 25 mg/day after 1 week. Patients not under spironolactone therapy due to limiting side effect (most often gynecomastia) were offered eplerenone, 25 mg daily. Other adjustments in medical therapy were left to the discretion of the attending physicians, although we recommended minimizing such changes to reduce variables, which could affect the EPC profile. Blood potassium, creatinine, and blood urea nitrogen levels were measured 1 week following therapy initiation. Patients were examined by a physician (TB, IM, TH, DM) and a nurse specializing in the management of CHF at baseline and after 8 weeks to determine functional capacity using a 6 minute walk test and the New York Heart Association (NYHA) scale.

BLOOD SAMPLING

Patients were tested twice: at baseline and after 8 weeks of MR antagonist therapy. Following overnight fasting, venous blood was drawn in heparinized tubes from an antecubital vein for EPC testing. Blood brain natriuretic peptide (BNP) levels were measured at baseline and after 8 weeks as a surrogate marker for CHF amelioration [14]. All samples were processed within 1 hour of blood collection.

CIRCULATING ENDOTHELIAL PROGENITOR CELL LEVEL AND FUNCTION

Circulating EPC levels were quantified by measurement of surface markers (VEGFR-2, CD34, and CD133) by flow cytometry. Functional aspects of EPCs were evaluated by measurement of colony-forming units (CFU) and MTT assay.

ISOLATION OF MONONUCLEAR CELLS

Peripheral mononuclear cells were fractionated using Ficoll density-gradient centrifugation (Lymphoprep, Alere Technologies AS, Oslo, Norway). The mononuclear cells were isolated and washed with phosphate-buffered saline after red cell lysis.

FLOW CYTOMETRY

Aliquots of peripheral mononuclear cells were incubated with monoclonal antibodies against vascular endothelial growth factor receptor 2 (VEGFR-2) (clone 89106, 10 ml to 10^5 cells, FITC labeled; R&D, Minneapolis, MN, USA), CD45-CYT5.5 (cy5 clone 7099, 10–100 ml cell suspension; Dako, Denmark), and either CD133 (clone AC133, 10 ml to 10^7 cells, PE-labeled; Miltenyi Biotech, Auburn, USA) or CD34 (clone AC136, 10 ml to 10^7 cells, PE-labeled; Miltenyi Biotech. Isotype-identical antibodies were used as controls. After incubation, cells were washed with phosphate-buffered saline and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, USA). Each analysis included 100,000 events, after selection for CD45-positive cells and exclusion of debris. Gated CD34- or CD133-positive cells were subsequently examined for the expression of VEGFR-2. Analyses were performed in duplicates. Results are presented as the percentage of peripheral mononuclear cells co-expressing either VEGFR-2 and CD133, or VEGFR-2 and CD34.

COLONY-FORMING UNIT QUANTIFICATION

Isolated peripheral mononuclear cells were re-suspended in medium 199 (Invitrogen, Carlsbad, CA, USA), supplemented with 20% fetal calf serum (Gibco BRL Life Tech, Gaithersburg, MD, USA), and plated on 6-well plates coated with human fibronectin at a concentration of 5 × 10^6 cells per well [3]. After 48 hours, the non-adherent cells were collected and replanted onto fibronectin-coated 24-well plates (10^6 cells per well). EPC colonies were counted using an inverted microscope 7 days after plating. As previously described, an EPC colony was defined as a cluster of at least 100 flat cells surrounding a cluster of rounded cells [3]. To confirm endothelial cell lineage, indirect immunostaining of randomly selected colonies was performed with fluorescently-labeled low-density lipoprotein (Dil-Acil-LDL) engulfment and with antibodies directed against CD31 (PECAM-1; Becton Dickinson, USA), Tie-2 (C-20; Biotechnology, Santa Cruz, CA, USA), and VE-cadhedrin. Results are expressed as the mean number of CFUs per well.

MTT ASSAY

The MTT assay was performed on samples from 12 randomly selected patients to evaluate the viability of the cultured EPCs [15]. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) measures mitochondrial activity in living cells. After 7 days of culture, MTT (Sigma, St. Louis, MO, USA) 1 mg/ml was added to the EPC medium culture, and incubated for an additional 3 to 4 hours. After the incubation, the medium was removed and the cells were solubilized in isopropanol. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT crystals, which can be dissolved in isopropanol. The amount of the dye released from the cells was measured with a spectrophotometer at 570 nm and subtracted background at 690 nm. An increase in the number of viable cells results in an increase in the amount of MTT formed and, therefore, in absorbance. Therefore, optical density is directly correlated with viable cell quantity [15].

STATISTICAL ANALYSIS

EPC parameters (flow cytometry determined levels, number of CFUs, and results of the functional assays) were not normally distributed, determined by the Shapiro–Wilk normality test. Therefore, the EPC data are presented as median and interquartile range (IQR) and comparisons were performed by two-tailed Wilcoxon matched-pair signed rank test (intra-group) or the Mann–Whitney–Wilcoxon test (inter-group). Other parameters in the study and clinical variables were normally distributed and therefore, are presented as mean ± standard deviation. Comparisons of the continuous normally distributed variables were performed by paired Student's *t*-tests. Categorical variables were compared using chi-square tests. All analyses were conducted using R: A language and environment for statistical computing, version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). $P < 0.05$ was considered statistically significant.

RESULTS

Twenty-eight patients in various stages of CHF were initially recruited. After exclusion of four patients (2 due to medical therapy non-compliance, 1 due to refusal of re-examination following the study termination, and 1 due to hospitalization for CHF decompensation during the study period), 24 were evaluated in the final analysis. Mean age was 66 ± 10 years, and the majority of the patients were male (85%). Cardiomyopathy was related to ischemic heart disease in 58% of the patients. Diabetes was present in 63% of patients. Mean ejection fraction was 30% ± 6%, and all patients were either in functional class II (58%) or III (42%). As indicated by the current guidelines for the management of CHF [16], all patients were prescribed beta blocker therapy and either ACE inhibitor (40%) or angiotensin receptor blocker (60%) therapy. Most patients were prescribed statin (80%), aspirin (90%), and furosemide (75%) therapy. Baseline characteristics are described in Table 1. Following recruitment, patients received 8 weeks of MR antagonist therapy with either spironolactone (66%) or eplerenone (33%) and were re-evaluated at the end of the trial period. While median BNP values declined from 318 pg/ml (IQR 80–677) at baseline to 156 pg/ml (IQR 60–309) at study termination ($P = 0.001$), 6 minute walk test length did not differ significantly between evaluations ($P = 0.5$). Self-reported functional class levels improved in 30% of patients (NYHA functional class declined from III to II in three patients, and from NYHA class II to class I in three patients) and remained unchanged in the rest.

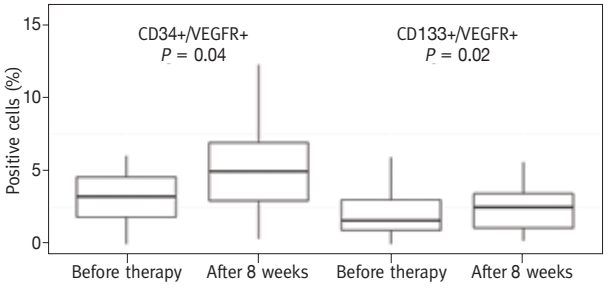
Figure 1 depicts the proportion of PMN cells that co-expressed VEGFR-2 and CD34, or VEGFR-2 and CD133, as determined by flow cytometry. A significant increase in the proportion of both VEGFR 2+/CD34+ cells (3.25% [IQR 1.8–4.6%] vs. 4.96% ([IQR 2.9–6.9%], $P = 0.04$) and VEGFR-2+/CD133+ cells (1.59% [IQR 0.93–3%] vs. 2.77% [IQR 1.1–3.45%], $P = 0.02$) was observed after MR antagonist therapy completion, reflecting higher levels of circulating EPCs. The choice of MR antagonist (eplerenone or spironolactone) did not impact the delta between post therapy and baseline EPC level (P = NS), nor did the presence or absence of ischemic heart disease (P = NS). The median number of EPC CFUs following 7 days of culture did not rise significantly after MR antagonist therapy completion (1.65 [IQR 1–2.8] vs. 1.8 [IQR 1.2–2.7]) or colonies per high power fiel, ($P = 0.42$) [Figure 2]. Figure 3 displays representative EPCs colonies before and after therapy. Viability of the cultured

Table 1. Patient baseline characteristics

	All patients N=24	Spironolactone therapy n=16	Eplerenone therapy n=8
Age, years	66 ± 10	66 ± 10	64 ± 11
Male gender	20 (85%)	13 (81%)	7 (88%)
Ischemic heart disease	14 (58%)	10 (63%)	4 (50%)
Diabetes mellitus	15 (63%)	10 (63%)	5 (63%)
Current smoking	2 (8.3%)	1 (6%)	1(12.5%)
Past smoking	13 (54%)	8 (50%)	5 (63%)
Hyperlipidemia	18 (75%)	14 (88%)	4 (50%)
Atrial fibrillation	5 (20%)	4 (25%)	1 (12.5%)
Hypertension	11 (46%)	7 (43%)	4 (50%)
Medical therapy			
Aspirin	21 (88%)	14 (88%)	7 (88%)
Warferin	5 (25%)	4 (25%)	1 (12.5%)
Furesomide	17 (71%)	11 (69%)	6 (75%)
Statin	20 (71%)	13 (81%)	7 (88%)
Ace inhibitor	10 (42%)	8 (50%)	2 (25%)
ARB	14 (58%)	8 (50%)	6 (75%)
Beta blocker	24 (100%)	16 (100%)	8 (100%)
Digoxin	3 (12.5%)	3 (19%)	0 (0%)
Nitrate	3 (12.5%)	3 (19%)	0 (0%)
NYHA class			
Class 2	14 (58%)	10 (63%)	4 (50%)
Class 3	10 (42%)	6 (37%)	4 (50%)

ARB = angiotensin receptor blocker, NYHA = New York Heart Association

Figure 1. Proportion of peripheral mononuclear cells that co-expressed vascular endothelial growth factor receptor 2 and CD133, or vascular endothelial growth factor receptor 2 and CD34, before therapy and after 8 weeks of mineralocorticoid antagonist therapy (n=24). Results expressed as median (25th–75th percentile)



VEGFR-2 = vascular endothelial growth factor receptor 2

Figure 2. Quantification of endothelial progenitor cell colonies following 7 days of culture before and after 8 weeks of mineralocorticoid antagonist therapy (n=24). Results expressed as median (25th–75th percentile). Representative EPCs colonies are depicted below the respective bars (magnification ×20)

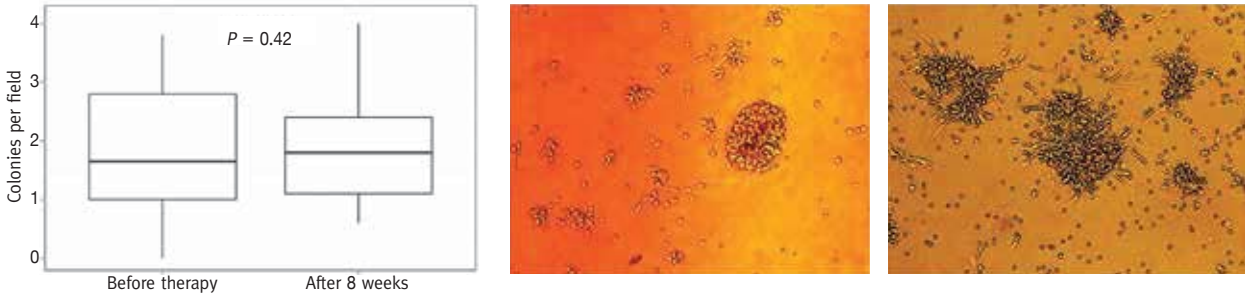
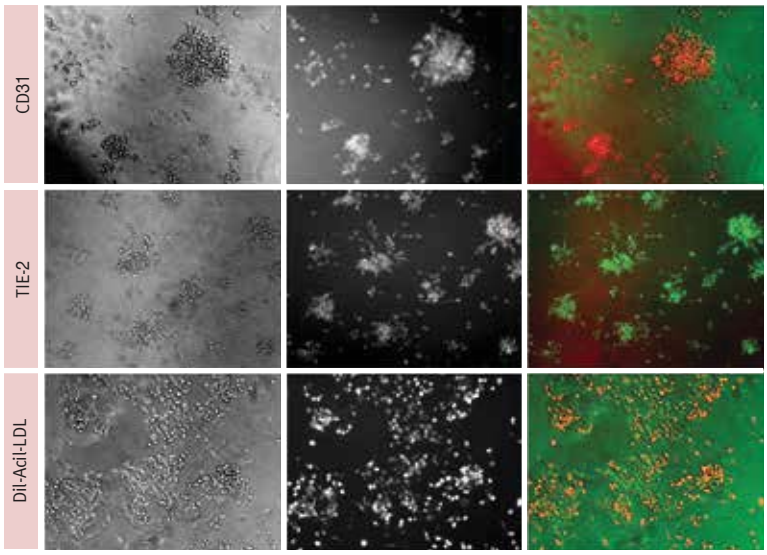


Figure 3. Representative EPCs colonies after 7 days of culture with positive uptake of Dil-Acil-LDL, and positive staining with antibodies directed against CD31 and Tie-2 to confirm endothelial cell lineage (magnification ×20)



Dil-Acil-LDL = fluorescently-labeled low-density lipoprotein

cells after therapy improved in comparison to baseline (as tested by the MTT assay), albeit not achieving statistical significance (P = NS).

DISSCUSION

Our results suggest that treatment with MR antagonists for 8 weeks leads to a rise in VEGFR-2+/CD133+ and VEGFR-2+/CD34+ cells in patients with established CHF, in conjunction with biochemical signs of CHF amelioration (a significant drop in BNP levels). However, functional aspects of EPCs did not significantly change following MR antagonist therapy.

Several previous trials have suggested that patients with CHF have significantly reduced levels of circulating EPCs compared with healthy individuals [17]. Nevertheless, other studies have observed a biphasic bone marrow pattern of response to CHF with increased peripheral recruitment of EPCs in early stages of CHF and reduced peripheral mobilization in NYHA class IV CHF [18,19]. A possible explanation for this phenomenon may be a surge in EPCs mobilization in response to endothelial damage in early stages of CHF with an exhaustion of progenitor cells in the advanced phases of the disease. In agreement, the baseline levels of peripheral EPCs in our cohort of NYHA class II and III patients were elevated compared to controls assessed in previous trials performed by our group [20]. Another possible explanation for the increased baseline recruitment of EPCs in the current trial may be the high rate of medical therapy with statins and ACE-inhibitors, which were previously shown to induce EPCs response in patients with CHF [21]. Interestingly, even though high EPC count were identified by flow cytometry, colony counts were relatively low and colonies less robust compared to controls assessed in previous trials performed by our group, probably reflecting a low viability and high senescence of EPCs due to heavy disease burden.

Large clinical trials have previously shown that MR blockade decreases mortality in cardiovascular disease [10,11] and ameliorates endothelial function in patients with CHF [9, 22]. While a cross-sectional trial had previously found a positive effect of eplerenone therapy on the number of EPCs in patients with CHF [23], to the best of our knowledge the current trial is the first prospective cohort study to evaluate EPCs response to MR antagonist therapy in patients with CHF. We found a significant rise in mobilization of both VEGFR-2+/CD34+ and VEGFR-2+/CD133+ cells, reflecting enhanced early EPCs mobilization. Although there was a signal for improved cell viability following MR antagonist therapy, and reflected by the MTT study, overall functionality of the cells as evaluated by their colony forming capacity was not improved. The increased EPCs mobilization may be explained by a direct effect of MR blockade on early EPCs, as early EPC were shown to express the MR [12,13] and in vitro studies demonstrated a reduction in circulating EPC number in response to aldosterone [12]. In vivo studies yielded similar results. Ladage and colleagues [24]

demonstrated significantly reduced circulating EPCs number in rats exposed to 28 days of aldosterone therapy compared to controls. Spironolactone antagonized this effect in part, leading to a twofold increase in EPCs number. Thum and co-authors [13] showed a qualitative rather than quantitative impairment of circulating EPCs by aldosterone, which was reflected by increased intracellular reactive oxygen species (ROS), decreased migration and differentiation potentials, and prevention of homing of early EPCs to implanted matrigel plugs. These effects were partially reversible by the selective MR antagonist eplerenone. Likewise, a clinical study on a small cohort of patients [13] with primary hyper-aldosteronism (n=10) demonstrated impaired EPCs function and migratory potential compared with controls without a significant difference in the numbers of circulating EPCs. Treatment of patients with primary hyper-aldosteronism with the MR antagonist spironolactone for 4–6 weeks significantly improved EPCs function [13]. Alternatively, the enhancement of EPCs mobilization may be a surrogate marker for CHF amelioration caused by MR antagonists rather than a direct effect of MR antagonists on EPCs consistent with prior trials demonstrating rise in EPCs mobilization in response to the amelioration of CHF during hospitalization [19,25]. We found no significant difference in EPCs rise after 8 weeks between patients who were treated with the selective MR antagonist eplerenone and those who received the none-selective antagonist spironolactone. However, this subgroup analysis was limited by the small sample size and further investigation is required.

The main limitation of the current study is the small sample size, which curtailed an effective comparison between spironolactone and eplerenone with respect to their effect on EPCs. It should therefore be regarded as a hypothesis generating preliminary study that requires further validation. Second, due to the nature of our cohort, patients were burdened with multiple background diseases and most were taking many medications at the time of recruitment, which hampered our ability to analyze the pure effect of MR antagonists on EPCs in CHF patients. However, we tried to eliminate the effects of other medications by restricting changes in pharmacotherapy during trial period to minimum.

CONCLUSIONS

The results of this preliminary study demonstrate that in patients with CHF, MR antagonist therapy leads to an increase in early circulating EPC numbers, without significant effect on cell function. These findings may account in part for the beneficial effects of MR antagonist therapy on heart failure pathophysiology and outcomes; however, further research in larger trials is needed.

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Capsule

Antitumor immunity

Immune checkpoint inhibitors induce durable tumor regressions in some, but not all, cancer patients. Understanding the mechanisms that determine tumor sensitivity to these drugs could potentially expand the number of patients who benefit. **Pan** et al. discovered that tumor cells in which a specific SWI/SNF chromatin remodeling complex had been experimentally inactivated were more sensitive to T cell-mediated killing. The cells were more responsive to interferon-γ, which led to increased secretion of cytokines that promote antitumor immunity. **Miao** and co-authors examined the genomic features of tumors from patients with metastatic renal cell carcinoma who had been treated with immune checkpoint inhibitors. Tumors harboring inactivating mutations in *PBRM*1, which encodes a subunit of the same SWI/SNF complex, were more likely to respond to the drugs.

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Eitan Israeli

Capsule

Prevention of tuberculosis in rhesus macaques by a cytomegalovirus-based vaccine

Despite widespread use of the bacille Calmette–Guérin (BCG) vaccine, tuberculosis (TB) remains a leading cause of global mortality from a single infectious agent (*Mycobacterium tuberculosis* [Mtb]). Over two independent Mtb challenge studies, **Hansen** and colleagues demonstrated that subcutaneous vaccination of rhesus macaques (RMs) with rhesus cytomegalovirus vectors encoding Mtb antigen inserts (RhCMV/TB), which elicit and maintain highly effector-differentiated, circulating and tissue-resident Mtb-specific $CD4^+$ and $CD8^+$ memory T cell responses can reduce the overall (pulmonary and extrapulmonary) extent of Mtb infection and disease by 68%, as compared to that in unvaccinated controls, after intrabronchial challenge with the Erdman strain of Mtb at approximately1 year after the first vaccination. Fourteen of 34 RhCMV/TB-vaccinated RMs (41%) across both studies showed no TB disease by computed tomography scans or at necropsy after challenge (as compared to 0 of 17 unvaccinated controls), and 10 of these RMs were Mtb-culture-negative for all tissues, an exceptional long-term vaccine effect in the RM challenge model with the Erdman strain of Mtb. These results suggest that complete vaccine-mediated immune control of highly pathogenic Mtb is possible if immune effector responses can intercept Mtb infection at its earliest stages.

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Capsule

Tracking inflammation in the colon

The therapeutic options for ulcerative colitis, a form of inflammatory bowel disease, are limited. **Lyons** and colleagues tracked gene expression, protein levels, and protein phosphorylation in individual animals in a mouse model of colitis. Computational analysis of the data sets identified discrepancies between transcriptomic and proteomic measurements and predicted that the kinase Pak1 mediated colonic inflammation. Treatment of mice with a pharmacological inhibitor of Pak1 ameliorated disease, highlighting the importance of proteomic measurement to the understanding of disease pathogenesis.

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