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# C-reactive protein subverts the myeloid lineage: implications for renal injury

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# C-REACTIVE PROTEIN SUBVERTS THE MYELOID LINEAGE: IMPLICATIONS FOR RENAL INJURY

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MICROBIOLOGY

ABSTRACT

C-reactive protein (CRP) is an evolutionarily conserved pattern recognition molecule that was first characterized for its participation in the acute phase response to bacterial infection. In the last 90 years, knowledge of CRP biology in innate immunity has expanded significantly. CRP is known to bind and activate the classical pathway of complement, to opsonize bacteria and inflamed tissue, and to modulate myeloid cell functions. CRP is normally present in the blood at low levels with its biosynthesis and serum concentration rapidly rises upon systemic inflammation. For example, high levels of serum CRP are found following acute kidney injury (AKI) in both humans and human CRP transgenic mice (CRPtg) undergoing a model of AKI wherein they experience worse outcomes than their WT counterparts. Additional work showed that during AKI, CRP mobilizes a population of leukocytes: myeloid derived cells with suppressor functions (MDSC) that appeared to be responsible for CRP's harmful effects.

My dissertation research sought to address how CRP modulates myeloid cell development, maturation, and function and whether MDSC actions could directly injure the renal epithelium, the major cell type damaged during AKI. I open with an extensive overview of CRP and how my dissertation project fits within the larger context of CRP research. Next, I introduce AKI epidemiology and our experimental understanding of its cellular pathology and myeloid cell involvement. I also included a brief overview of

myelopoiesis as this is essential for understanding MDSC biology. My chapters are organized in order to show i) CRP regulates dendritic cell development, function, maturation, and its consequences in autoimmunity, ii) CRP promotes MDSC development and suppressive functions, and iii) MDSCs impair renal epithelial cell cycling. I end with a discussion of a newly formed thesis: CRP is an evolutionarily conserved molecule that modulates the functional phenotype of myeloid cells during their development from bone marrow progenitors. Further, I overview known CRP effects on differentiated myeloid cells, its roles in autoimmunity, a hypothesis of its effects in AKI in light of the data presented herein, and the future of CRP myeloid modulation in cancer.

Keywords: C-reactive protein, myelopoiesis, acute kidney injury, innate immunity, myeloid, pentraxin

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# INTRODUCTION

## C-Reactive Protein

### *Historical Overview*

Pentraxins are a class of pattern recognition molecules that are highly evolutionarily conserved in both sequence – genomic and peptide – and function. The word 'pentraxin' derived from the Greek words *penta* (five) and *ragos* (berries) aptly describe their structure: five globular monomers non-covalently bound together in a planar ring with a central pore (Figure 1). The prototypical pentraxin, human C-reactive protein (CRP), was discovered for its ability to react to the "C fraction" of *Streptococcus pneumoniae*[1], specifically via the phosphocholine residues of the C-polysaccharide (C fraction) of the pneumococcal cell wall. Also, the high "C-precipitin" blood levels seen in pneumonia patients at hospital admission drastically decreased coincidently with the resolution of their febrile period – the first published observation of CRP's characteristic rise and fall during what was termed the 'acute phase response'. Thereafter, other groups began testing the blood of patients with Gram positive and negative bacterial infections, rheumatic fever, and various diseases; uniformly observing the presence of the "C-reactive" substance in titers that were well above the antibody titers found within the same patient[2-4]. Importantly, Ash found C-precipitin in the blood of a six month old infant, i.e. early in life before significant development of acquired immunity[2]. These foundational observations demonstrated that CRP likely has physiologically functions distinct from antibodies.

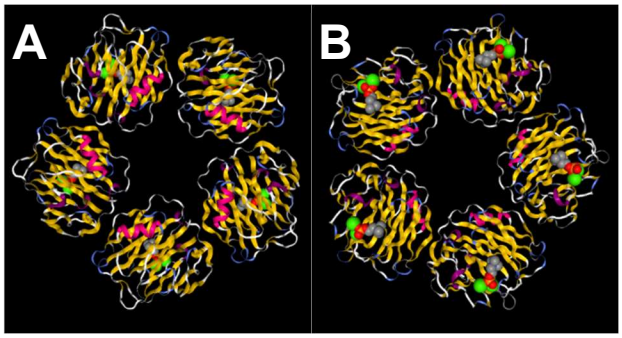



**Figure 1. C-reactive protein is a pentameric ring with two distinct faces.** (A) The A face has an α-helix (fuchsia). (B) Each monomer binds two $Ca^{2+}$ ions (green space fill) that coordinate phosphocholine binding (gray, red, orange space filled molecules). Image of 1B09[5, 6] created with NGL[7] accessed on PDB[8-10].

It was also noted early that those patients that perished from infection maintained high titers of the C-reactive substance. Additionally, Abernathy and Avery showed that in both humans and cynomolgus monkeys the C-reactive substance is found in serum, plasma, and pleural effusions and that its precipitating function can be inactivated upon calcium chelation (e.g. with oxalate, citrate) and restored by the addition of $CaCl_2$[11]. Subsequent studies determined that the C-reactive substance was a protein[11, 12]. At this point, the nomenclature 'C-reactive protein' (CRP) was adopted and CRP in other species were discovered, described, and purified based on their identity as a multimeric protein able to react with C-polysaccharide in a $Ca^{2+}$-dependent manner that are important for host-pathogen interaction[13].

The potential use of CRP as a marker of early and ongoing infection was quickly recognized as early as April 1941 and later proven experimentally[14, 15]. From that point onwards, the blood of patients were measured for CRP and its presence used to aid in clinical diagnoses and to monitor treatment. As such, early on CRP was labeled a "biomarker of inflammation" and even today CRP levels are factored into disease risk assessment guidelines.

Initially using the standard approaches then available (nephelometry, immunodiffusion, precipitation), CRP could only be detected when its levels were elevated in the serum, such as in patients experiencing acute or severe disease, and could not be found in the blood of healthy individuals. As more sensitive and precise techniques were developed from the late 1970s onward[16], it became apparent that trace amounts of CRP were also present in the serum of healthy patients, both male and female. In fact, it was noted early on that increased CRP levels correlated with increased age, a phenomenon that is not well understood nor extensively researched even today[15-20]. Observing CRP in the blood of healthy humans challenged the paradigm that CRP was simply an opsonin during infection, raising the possibility it was also a mediator of immunity. Indeed, today we know that high baseline expression of CRP in ostensibly healthy, and not their underlying inflammation nor dyslipidemia, is the most robust predictor of future adverse coronary events[21, 22]. Despite this, the clinical utility of CRP is still wanting as any positive association of CRP to disease is oft misinterpreted as causal or something "to be managed". Today we know that CRP plays an important role as an adaptor of immunological reactions between innate and adaptive immunity.

In the mid-1970s researchers began exploring CRP's biochemical and structural features, its effects on leukocytes, and its role in immunity[23-25]. With the amino acid sequencing of human CRP came the recognition of its high evolutionary conservation and similarity to other immune molecules such as immunoglobulins, C3a, and serum amyloid P component (SAP)[26]. It was these studies that led to the coining of '*pentraxin*' to define this class of proteins, for which CRP is the prototype[27]. Seminal studies done in rabbits showed that the liver is the main site of production acute phase proteins (APP), including

CRP. This finding, along with the mapping of human *CRP* to chromosome 1[24], led to studies of the transcriptional control of CRP expression by hepatic factors[28] and the APR mediators IL-6 and IL-1β[29, 30]. Today, transcription regulation of *CRP* is recognized as the main regulatory mechanism for controlling CRP blood levels.

Evidence that CRP was a pattern recognition molecule were first evident when CRP was found to bind phosphocholine moieties on structures in addition to C-polysaccharide, such as the apoptotic cell membrane. In addition to its role as an opsonin, CRP was also found to bind C1q and to activate the classical complement pathway. CRP can also bind small ribonucleoprotein particles[31, 32], a mechanism thought to contribute to autoimmunity directed against nuclear antigens as seen in lupus. Not only does CRP bind ligand it can also can alter leukocyte functions, like phagocytosis via receptor binding, including various Fcγ receptors (FcγRI, FcγRIIA/B, and FcγRIII), Fcα receptor I, and the oxidized LDL receptor (LOX-1)[33, 34]. These varied ligand and receptor interactions suggest that CRP participates beyond infection and is an important soluble mediator of innate immunity[35, 36].

The new-found ease of measuring serum CRP with its clear differences from baseline to APR expression, led to serum CRP being readily adopted into other clinical settings; CRP experienced a renaissance and a publication boom as new disease associations were reported around the turn of the century (Figure 2). From cardiovascular disease to cancer to acute kidney injury, high levels of CRP is correlated with worse prognosis. Conversely, high CRP levels in autoimmune disease, such as multiple sclerosis, arthritis, and lupus, predict better patient outcomes. Also, in both humans and human CRP transgenic mice (CRPtg), there is sexual dimorphism in CRP expression.

Still up to the mid-20th century, few studies investigated causation of CRP in disease pathogenesis. Furthermore, *CRP* genomic variations that influence CRP expression at baseline and/or during APR are not fully appreciated. Thus, despite this wealth information, the "big picture" of CRP's contribution to human health is still not fully considered, particularly within the clinical sphere.

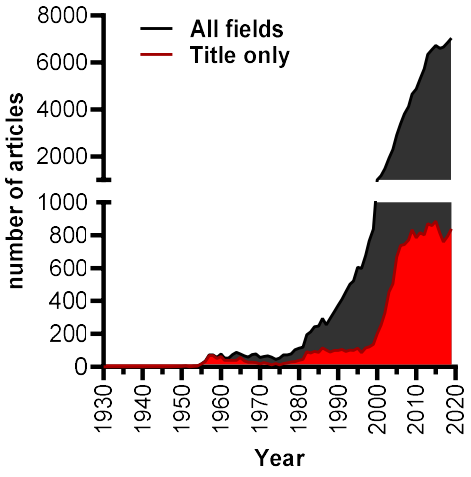



**Figure 2. The number of articles referencing CRP.** A PubMed search using the terms "C reactive protein" OR "C-reactive protein" OR "CRP" in either "All fields" or "Title only" reveal three modalities to the number of CRP-related publications. The first published description of the "C precipitin" substance in 1930. The uptick in publications was largely fed by a seminal conference in November 1982 that coalesced disparate findings on CRP and its role in the acute phase response (Kushner I., et al. 1982) and a boom in research 2000 owing to CRP transgenic animal models and the use of CRP as a clinical biomarker of inflammation called high-sensitivity CRP (hs-CRP) which detects low CRP levels[38]. The ease in hs-CRP testing led to its many clinical associations and reporting therein far exceeds the publications investigating CRP biology.

*The Acute Phase Response:*
*A Brief Overview and its Relationship to the Phenomenon of Inflammation*

The approach taken in the following introductory subsections to CRP is largely a compare-and-contrast between its biology during the acute phase response (APR; i.e. when CRP levels are high) versus baseline (i.e. when there is no overt systemic inflammatory stimulus and CRP levels are low). This serves two purposes: i) the early and seminal discoveries of CRP biology were in the clinical and experimental context of an APR and ii) to clearly show that CRP has important and less well defined roles during homeostasis (i.e. outside of the APR).

The acute phase response (APR) is the body's reaction to systemic inflammation, and it is characterized by alterations in the hepatic expression of several non-protein (e.g. hormones, lipids) and protein (i.e. APP) blood constituents and consequent changes in behavior, physiology, biochemistry, and metabolism[39]. Clinically, the APR is defined by a change of 25% or more in the blood concentration of acute phase proteins (APP), the prototype being CRP which itself can increase anywhere from 300- to 1000-fold[16, 40]. Changes in hepatic APP expression typically are stimulated by cytokines produced from local immune reaction(s). The major inducer of the APR is IL-6, working in concert with IL-1β, TNFα, IFNγ, and TGF-β. In addition to changes in APP concentrations, other systemic changes occur that will not be discussed here (for intriguing reviews see[39, 41, 42]). Oftentimes, the APR is used synonymously with inflammation but there are important distinctions; principally, inflammation can occur without triggering an APR and inflammation is part of the constellation of events preceding and concomitant with the APR. Indeed, all of the hallmarks of inflammation are present during an APR: *calor*, *rubor*, *dolor*, and *tumor*.

'Inflammation' suffers from similar common misuse in contexts where not all the cardinal signs are found: such as with "low-grade" or "meta" inflammation or that observed over a long period of time, called 'chronic' inflammation (other terminology has been proposed as well [43]). The association of CRP blood concentrations with a wide spectrum of these inflammatory conditions likely reflects a bimodal biological response (Figure 3): during chronic/low-grade inflammation, elevations of CRP expression are more likely due to genetic variations (discussed below); whereas in acute inflammatory contexts the serum levels of CRP reflect an environmentally driven response. In the latter

case, the genetic contribution to CRP levels is likely "washed out" by the robustness of the CRP expression induced by an APR (discussed below).

Within this dissertation, i) 'inflammation' refers to contexts wherein the four cardinal signs are observed and ii) the APR is a liver response, principally in APP expression, that results in systemic changes. These definitions are necessary in the following discussion of CRP biology, especially in its transcriptional regulation, as an explanation for its clinical associations.

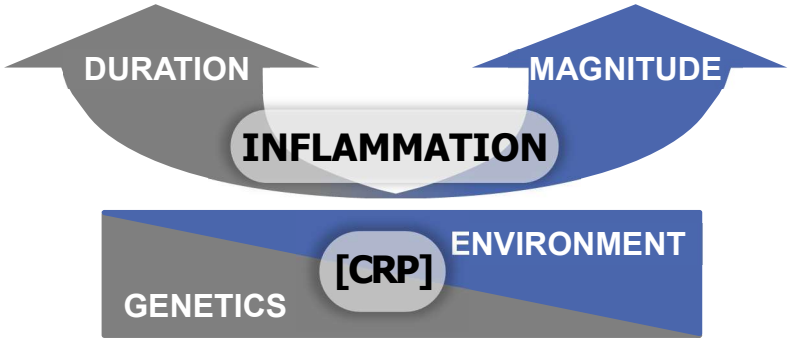



**Figure 3. Inflammation and C-reactive protein.**Today, the term inflammation is oft used in a range of contexts, including in the presence or absence of overt challenge to the host. Acute settings (i.e. scenarios trending toward the right with a high magnitude of response over a short period) have the characteristic cardinal hallmarks of inflammation. In contrast, chronic, low-grade 'inflammation' (i.e. minute responses over a long duration) does not contain all signs of inflammation (*dolor*, *rubor*, *calor,* and *tumor*). Concordantly, CRP concentrations are frequently utilized as a clinical readout of inflammatory processes. However, the increase of CRP levels vary from an individual's genetics over their lifespan to rapid increases in response to injurious scenarios. Therefore, heightened CRP levels obfuscate the underlying sequelae and must be taken within the inflammatory context.

*CRP Gene Structure*

Soon after its discovery, it was realized that serum CRP could be found in healthy individuals, but CRP was primarily measured for clinical prognosis of ongoing inflammation during acute infection. Therefore, researchers sought to determine i) the regulatory mechanism for basal CRP expression and ii) how does infection induce higher levels of CRP expression. From the early 1980s to the mid-1990s these questions led

researchers to study the *CRP* promoter region. Most of the ensuring genetic and molecular discoveries were made using rabbit or human hepatocytes or rabbits; later studies confirmed many of these findings in human CRP transgenic mice that were generated in the mid-1990s.

Notably so far there is no known case of complete CRP deficiency in humans. Furthermore, almost every organism with a sequenced genome has one or more pentraxin encoded, with CRP found in evolutionarily distant species such as the Atlantic horseshoe crab (*Limulus polyphemus*). Together, these observations highlight the importance of this serum molecule for life in general.

Located on chromosome 1q23.2, the human CRP gene locus includes a promoter region, a protein coding region, and a pseudogene [24]. The full mRNA transcript contains a small 5'UTR, 2 exons separated by an intron, and a large 3'UTR (Figure 4). Note that exon 1 is quite small with only seven nucleotides (nt) that are amino acid (aa) encoding; the first two nt of exon 2 encode the completion of a codon. Translation of the mRNA yields a 224 aa peptide chain that includes an 18 aa leader peptide; the mature CRP monomer (206 aa) is post-translationally assembled into a pentamer.

The human CRP pseudogene (*CRPP1*; 624 nt) is 6,688 nt downstream of *CRP*. When aligned to *CRP*, the sequence homology of *CRPP1* ranges from 50 - 80% (*CRP* +649 to 1020, i.e. exon 2)[44]. Within *CRPP1* several missense and nonsense codons were found within the three possible open reading frames indicating; therefore, it is unlikely

that if transcribed, translation would produce stable peptide products. However, gene expression data from CRPtg mice suggest that *CRPP1* is likely the result of a gene duplication event and is retained due to its negative regulation on *CRP* expression.

The genomic sequences important for CRP expression were determined using human hepatocyte cell lines (e.g. HepG2, Hep3B), primary hepatocytes (rabbit, mouse, human), and most eloquently in human CRP transgenic mice (CRPtg). Early experiments using hepatocytes identified a proximal promoter needed for CRP expression in response to APR stimuli[45]. However, this region was expanded following *in vivo* studies in CRP transgenic mice generated with varying constructs. CRPtg mice generated with a large construct of the human CRP gene (+17 kb to -13.6 kb) showed normal expression compared to CRPtg mice generated from constructs containing minimal 5' and 3' sequences (higher expression), missing the CRP pseudogene (higher expression), or disruption of the poly(A) signal (no expression)[46]. These mice confirmed *in vitro* data

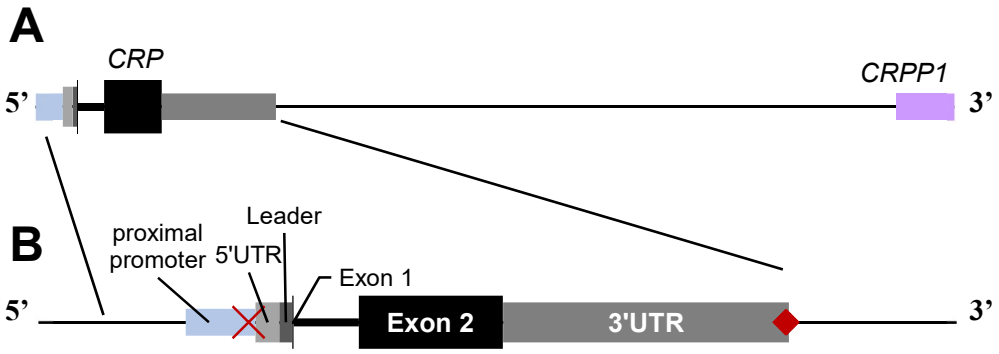



**Figure 4. The human C-reactive protein gene.**(A) Downstream of CRP is the CRP pseudo-gene (*CRPP1*, lilac box) that is not known to produce a stable gene product but is likely involved in *cis*-acting regulation of basal *CRP* expression. (B) The proximal promoter of *CRP* (light blue) is typically mapped as 300 nt upstream of the transcription start site, including the TATA binding site at -29 to -26 (X). The short 5'UTR (light gray box, left) precedes the coding sequence for the leader peptide (18 aa; dark gray) that is later cleaved in the ER. Exon 1 encodes the first 3 aa of mature CRP (thin black line) and is immediately followed by the intron. The remainder of CRP is encoded by exon 2 (black box, right). *CRP* has a long 3'UTR sequence (light gray box, right) with a poly(A) signal (◆). Regions are drawn to scale.

showing the elements needed for hepatic constitutive expression are found close to *CRP* and elucidated the impact of the large 3' and 5' sequences needed for constitutive repression of its expression. More specifically, the promoter response elements can be placed into two categories: hepatic-specific and acute phase response inducible. Both regulatory mechanisms work coordinately to allow for liver-specific and inflammatory-stimulated expression. The proximal CRP promoter is typically defined at the 300 nt upstream of the transcription start site and can be roughly divided into two regions: an upstream region and a TATA box-proximal region centered around the -43 position that is crucial for NF-κB binding (Figure 5).

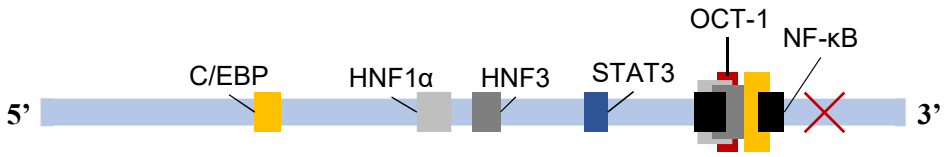



**Figure 5. Transcription factor binding sites along the CRP proximal promoter.** Hepatic transcription factors (HNF1α, HNF3, gray boxes) regulate constitutive expression of *CRP* by hepatocytes. In response to APR induction, primarily IL-6 and IL-1β, the transcription factors C/EBP (yellow boxes), NF-κB (black boxes), and STAT3 (blue box) bind. Note that C/EBP and NF-κB compete at the site close to the TATA box (red X) and the transcription start site (right end of green box). One repressor, OCT-1 (red box), is known to bind and compete at this same location.

*Hepatic-specific CRP expression.* Soon after CRP's discovery it was found to be secreted by the liver, specifically by hepatocytes. Hepatocytes are the major secretors of other acute phase proteins, including albumin, serum amyloid P component (SAP, the other major pentraxin), ferritin, transferrin, etc. As such, many of the studies that teased out the transcription factor binding sites in the *CRP* promoter utilized hepatocyte cell lines (i.e. Hep2 and Hep3B). Indeed, in 1990 two papers from Ciliberto's group identified a binding site for the hepatic nuclear factor 1 homeobox A (HNF1α; TCF1; H-APF-1)

within the *CRP* promoter and later identified an HNF3 consensus sequence that is assumed to aid HNF1α with liver-specific expression. HNF1α promotes *CRP* expression which can be augmented by IL-6 and IL-1β stimulation.

In humans, rabbits, rats, and CRPtg mice, hepatic production is also the major source of *baseline* serum CRP. This is clinically relevant as exemplified by patients with maturity onset diabetes of the young (MODY), a disease driven by decreased HNF1α function; these patients have decreased CRP expression and their serum CRP levels can be used to distinguish MODY-HNF1α from other forms of MODY and diabetes[47]. It is possible that reduced CRP seen in HNF1α MODY is a cause rather than a consequence of reduced HNF1α triggered transcription of CRP. In fact, our laboratory found that CRP knockout mice ($CRP^{-/-}$) have a MODY phenotype: at ≤5 weeks of age $CRP^{-/-}$ mice have fasting blood glucose levels similar to wild type mice (unpublished data). However, at maturity (≥ 6 weeks) $CRP^{-/-}$ mice have an increase in their fasting blood glucose levels that can be exacerbated by a high fat diet (unpublished data).

*Acute phase response induction of CRP expression.* As noted earlier, the rapid elevation of CRP occurs during the APR. Of the many cytokines released during the APR, IL-6 and IL-1β, play the predominant role in upregulation of CRP.

*IL-6 induced CRP expression.* IL-6, the main driver of the APR, is the major inducer of CRP expression. Early studies reported two IL-6 responsive regions that were dissected to reveal binding sites for the transcription factors STAT3 and C/EBP (β and δ isoforms)[29, 30, 48]. C/EBPβ basally binds, but its binding capacity is increased upon cytokine exposure[30].

To confirm the involvement of IL-6 for CRP expression *in vivo*, CRPtg mice were crossed with IL-6 knockout mice (IL-$6^{-/-}$). In the CRPtg IL-$6^{-/-}$ mice, the human CRP transgene was basally expressed similar to CRPtg mice. In CRPtg $IL6^{-/-}$ mice however, exogenous IL-6 did not stimulate CRP upregulation whereas exogenous IL-1β did. Furthermore, these studies also showed that in CRPtg $IL6^{-/-}$ mice, IL-1β did not synergize with the IL-6 induced upregulation of CRP during LPS-induced and sterile inflammation. It is unlikely that during an authentic APR there would be production of only one cytokine therefore investigating individual cytokine involvement is likely moot.

*IL-1β induced CRP expression.* While studies on IL-6-induced CRP expression were ongoing, it was in fact the "endogenous" or "leukocytic" pyrogen, later named IL-1β, that was initially identified as an inducer of CRP expression[49]. IL-1β alone cannot or minimally induces CRP expression by hepatocytes but with dual exposure (IL-6+IL-1β) the amount of CRP produced is greater than either cytokine alone[50]. IL-1β activates both C/EBPβ and NF-κB (the transcription factors are known to physically interact). As such, it is proposed that IL-1β merely enhances the IL-6 induced CRP expression by increasing transcription of C/EBPδ or through promoting c-Fos binding both C/EBPβ to HNF-1α binding on the CRP promoter.

*Other regulators of CRP expression.* There are disparate reports of other molecules regulating CRP expression, with some stimulating hepatic expression and extra-hepatic sources; a few will be highlighted here. While there is no direct evidence, it is assumed that other cytokines involved in the APR are also inducers of CRP expression by the liver (e.g. IFNγ, TNFα) [51]. Furthermore, it is no surprise that CRP expression can be induced by leukemia inhibitory factor and oncostatin M, other members of the IL-6

family[52,53]. Other stimulators of CRP expression include hormones[54-59], other cytokines[60], complement activation products[61], and other signaling molecules to signal hepatocytes or other non-hepatic cell types[62-64].

*Sex based differences in CRP expression.* As mentioned above there is sex-based difference of CRP blood levels in humans and CRPtg mice. While studying its IL-6 responsiveness, Szalai et al. showed that human *CRP* was subject to sexual dimorphism in CRPtg mice challenged with lethal doses of LPS: male CRPtg mice had increased survival and lower bacteremia compared to CRPtg females[57]. Sex-based differences in CRP expression was also shown in humans[65, 66]. Based on CRPtg studies and human genetic associations (discussed below) it is likely that these sex-based differences are due to transcriptional control by hormone response elements (HRE) in the human CRP intron.

*CRP Population Genetics*

With the rise of the high-sensitivity CRP clinical assay (i.e. the high-throughput assay of low serum CRP levels) came the large-population based realization that baseline CRP level is a robust marker for cardiovascular disease. Other specialties have also investigated CRP levels as a predictor or marker of pathology, particularly autoimmune and rheumatic diseases with known inflammatory components. Soon after its introduction, genomic sequencing also seeped into clinical and experimental studies of CRP. Many groups worldwide sought to determine whether genomic variations in *CRP* exist and if they contribute to changes in baseline CRP expression; additionally, researchers sought to determine whether a genomic variation distinguished active disease processes or merely reflected the inflammatory response to the disease or comorbidities.

Several hundred genomic variations for *CRP* and its locus are digitally indexed and a systematic resequencing of the *CRP* locus described 40 SNPs and 29 different haplotypes, with the diversity highest in African Americans[67]. Several reviews and meta-analyses describe anywhere from 5 to 29 haplotypes in cohorts of patients with lupus, arthritis, or cardiovascular disease spanning African Americans, Caucasians, Han Chinese, Filipinos, and others. However, of these many *CRP* SNPs indexed in the National Center for Biotechnology Information (NCBI), only 18 have been reported in the primary scientific literature (Figure 6). Importantly, none of the SNPs investigated are reported to alter the protein structure nor ligand, complement, or receptor binding ability of CRP. The majority of studies investigate SNPs within the CRP promoter (light blue box, Figure 6), likely reflecting the biological importance of transcriptional control for CRP expression and notably, the *CRP* promoter is in linkage with its intron, exon 2, and 3' UTR[68, 69]. For a more detailed summary of *CRP* SNPs and their respective association with disease, see these reviews[70-72].

To date, the only SNP with demonstrated functionality is the triallelic rs3091244. Szalai et al. reported that the rs3091244 *A* minor allele forms a complete consensus

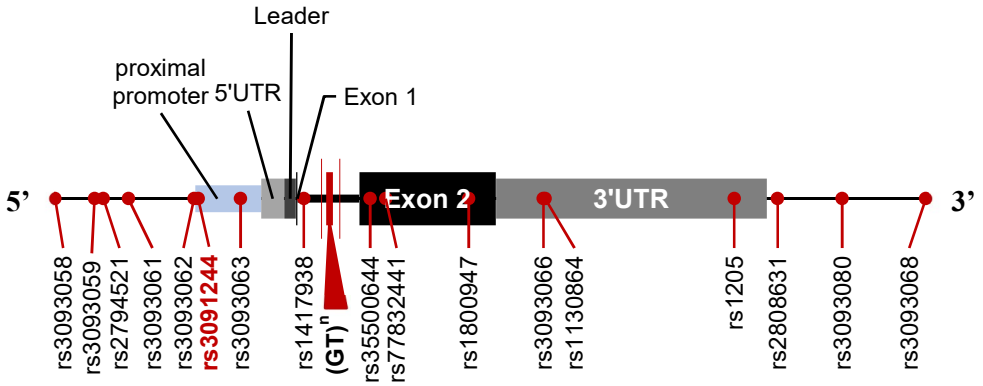



**Figure 6. Genetic polymorphisms in the *CRP* locus.** Commonly studied are 18 SNPs (●) and a microsatellite guanine thymine repeat ((GT)$^n$). Within the proximal promoter (light blue box) is the only SNP with known functional consequence, rs2091244 (red text). Depicted is a GT$^{16}$ flanked by the HRE-3 sites (long red vertical lines) thought to regulate transcription to exon 2.

sequence for upstream stimulatory factor-1 (USF-1), increased *CRP* promoter activity, and rs3091244 *AA* homozygotes have increased baseline serum CRP[73]. Similarly, the complement protein C4, which also rapidly rises in concentration during an APR, has a promoter E-box element to which USF-1 binds[74]. Thus, it is likely that USF-1 is a transcription factor that binds several APP promoters (the rs3091244 *A CRP* promoter).

Goldman et al. identified the first human *CRP* polymorphism, a microsatellite guanine thymine repeat (denoted $(GT)^n$) within the intron[44]. A microsatellite is defined by a short (1 – 6) nucleotide repeats ($\geq 9$)[75] and, in most higher order vertebrates, the most common intronic dinucleotide repeat are $(GT)^n$ repeats [76]. I propose that the *CRP* intronic $(GT)^n$ modulates the efficiency of transcription, and therefore baseline CRP expression, based on three observations: i) $(GT)^n$ adopt a Z-DNA conformation, ii) flanking the $(GT)^n$ is a predicted consensus sequence for the hormone response element-3 (HRE-3), and iii) intron $(GT)^n$ influence RNA processing.

It is known that $(GT)^n$ adopt Z-DNA conformation that is left-handed with 12 base pairs per helix turn[77]. If this logic is applied to the sequenced $(GT)^n$ alleles (which range from 9 to 25 dinucleotide repeats[78], only three alleles allow for complete helix turns (Appendix A, Table B). Notably, the $GT^{18}$ allele allows for 3 helix turns and in the report by Szalai et al, $GT^{18}$ associates with high baseline serum levels of CRP[78]. Therefore, the number of helix turns per $(GT)^n$ could influence *CRP* transcription.

Additionally, Szalai et al. proposed that the $(GT)^n$ alters the orientation of the predicted HRE-3 half sites[73]. The HRE-3 consensus sequence AGAACAxxxTGTTC is similarly found in the CRP intron as $AGAACAx_{17}(GT)^nx_{26}TGTTTC$. The possibility of an altered HRE-3 orientation may account for the known sexual dimorphism of CRP

expression, i.e. for both humans and CRPtg mice the males express higher CRP (at baseline and during an APR). Appendix A, Supplemental Table B predicts the total number of helix turns within the HRE-3 dyad, assuming 10 base pairs per helix turn in the intervening sequence (i.e. 44 base pairs). Interestingly, $GT^{16}$ and $GT^{21}$ allow for almost complete helix revolutions (7.1 and 7.9, respectively). Furthermore, Szalai et al. showed that $GT^{16}$ and $GT^{21}$ are the most common alleles in Caucasians and correlate with lower CRP blood levels[73]. However, it remains to be investigated whether there are sex differences in baseline CRP levels for a given $(GT)^n$ allele.

In other human gene introns, the number of GT repeats modulate splicing into mRNA[79, 80]. In *CRP*, the codon sequence for aspartic acid (the third aa of the mature CRP monomer) is encoded by exon 1 and exon 2; thus, splicing must properly and precisely occur for fidelity and sense of the *CRP* mRNA. Therefore, it is likely that the length of the intron (i.e. the number of GT repeats) could either promote or hinder the splicing machinery and regulate *CRP* expression efficiency.

These observations strongly suggest that the $(GT)^n$ polymorphism should correlate with baseline CRP expression. Several explanations offer mechanistic explanations that could function synergistically. For instance, during baseline conditions (i.e. not during an APR that would recruit additional transcription factors, as discussed elsewhere in this dissertation) the $(GT)^n$ adopts a Z-DNA conformation, bound by nucleosomes or not[77]. GT repeats of 18 or 20 could encode an immature RNA strand with an ideal length for the splicing machinery, thereby increasing the efficiency of CRP mRNA production. Conversely, $GT^{16}$ and $GT^{21}$ would be less ideal and thus decrease RNA splicing efficiency. Additionally, $GT^{16}$ and $GT^{21}$ allele would coordinate the HRE-3

half sites thereby allowing an unidentified repressor to bind to which the $GT^{18}$ allele would disrupt. Testosterone (either directly or through signaling cascades) would remove the repressor, in effect enhancing the ability of transcription machinery to proceed. These mechanisms would most likely be operant during baseline conditions. The highly responsive transcription machinery recruited during an APR would likely overwhelm what basally occupies the CRP locus.

*CRP Protein Structure*

The characteristic annular pentraxin structure of human CRP was first revealed by negative electron microscopy[26]. Nearly 20 years later, a full X-ray crystallographic structure of human CRP was resolved,[81] although partial human CRP crystals were previously generated[82]. Subsequently, human CRP was resolved with its typifying ligand phosphocholine[6] and without calcium[83]. These structures and mutagenesis studies were the basis for modeling how CRP bound complement C1q and Fcγ receptors[6, 84, 85]. Below is a broad description of CRP followed by the structure and function relationship of CRP.

As stated previously, CRP consists of five, non-covalently bonded monomers arranged in a ring with a central pore (Figure 1). From the 224 aa precursor peptide, an 18 aa leader peptide (negatively numbered amino acids, Figure 7) is cleaved in the ER. The resulting 206 aa peptide chain is folded into two antiparallel β sheets in a flattened jellyroll topology with a diameter of 36 Å and an intrasubunit disulfide bond formed by two half cystines (yellow highlights, Figure 7**Error! Reference source not found.**). The five monomers are held together by three salt bridges and assembled at a slight angle (15 – 20°). The resulting CRP pentamer inner and outer diameters roughly measure at 102 Å and 30 Å, respectively. The topology of CRP is maintained both in the presence or

absence of $Ca^{2+}$ and when bound to phosphocholine[6, 81, 83]. However, there is some findings that suggest when bound to a phospholipid bilayer the monomers flatten out due to the flexibility of the intersubunit disulfide bonds[86].

*The A face.* The A face of CRP has a cleft partially formed by an α-helix (underlined residues, Figure 7) that faces towards the central pore. The furrow contains positive charges on the surface with negative charges lining the bottom; it is narrow and deep at the center of the monomer but widens and becomes shallow at the central pore. The furrow topology is utilized for C1q and FcγR binding at a 1:1 stoichiometry[84, 87]. One of the globular head domains of C1q binds in the shallow end of the cleft of two monomers thereby sitting within the central pore of a single CRP pentamer[87, 88]. It is thought that multivalent binding of the globular heads of C1q across several CRP pentamers, aggregated for example, on apoptotic cell membranes, would allow for activation of C1q[86]. Interestingly, although CRP can activate C1q, CRP prevents assembly of the terminal membrane complement attack complex[89, 90]. As for FcR binding, one immuno-globulin domain of an FcγR use the α-helix residues along the

```
  -10  -1
MEKLLCFLVL TSLSHAFG
  10   20   30   40   50
QTDMSRKAFV FPKESDTSYV SLKAPLTKPL KAFTVCLHFY TELSSTRGYS
  60   70   80   90  100
IFSYATKRQD NEILIFWSKD IGYSFTVGGS EILFEVPEVT VAPVHICTSW
  110  120  130  140  150
ESASGIVEFW VDGKPRVRKS LKKGYTVGAE ASIILGQEQD SFGGNFEGSQ
  160  170  180   190  200
SLVGDIGNVN MWDFVLSPDE INTIYLGGPF SPNVLNWRAL KYEVQGEVFT
 206
KPQLWP
```

**Figure 7. CRP amino acid sequence.** A leader peptide (18 aa; negative positions, top row) is cleaved yielding a mature 206 aa monomer. RNA splicing must precisely occur for the mRNA to encode the complete codon for the third aa, aspartic acid (cyan highlight). The intrasubunit disulfide bond formed by two half cystines (yellow highlight). Key residues have been identified in the coordination of binding two $Ca^{2+}$ ions per monomer (green highlights). A nuclear localization motif (underlined residues) is correlated with CRP biological roles in nuclear autoantigen regulation. CRP contains one alpha helix (double underline).

furrow to bind two opposing monomers, resulting in CRP "laying" across the upward facing receptor[85]. CRP binding C1q or FcγR does not significantly change the conformation of the pentamer thus it is suggested that while the A face is engaged, the B face can concomitantly bind ligand.

*The B face.* On the binding (B) face, a shallow $Ca^{2+}$ binding pocket is roughly in the center of each monomer. This pocket is best characterized by its ability to bind phosphocholine (PC) wherein two $Ca^{2+}$ ions coordinate the negatively charged phosphate group while Glu81 interacts with the choline moiety[6, 81]. Thus, CRP can bind PC-bearing structures, including PC cell wall constituents, apoptotic cell membranes, lecithin, sphingomyelin[1, 89]. Interestingly, pentraxins binding to PC is used to characterize CRP molecules while binding phosphoethanolamine is used to define serum amyloid P component (SAP) molecules. Thus, the PC-binding property has allowed for consistent identification of CRP across species.

*Evolutionary Conservation of CRP*

CRP is an ancient innate pattern recognition molecule that is found even in invertebrates, such as the Atlantic horseshoe crab (bottom sequence, Figure 8). Interestingly, across orthologs from 104 species there is high conservation of the half-cystines involved in the intrasubunit disulfide bond and the residues involved in $Ca^{2+}$ ion binding (Figure 8). This suggests that the monomer structure and the overall PC-binding ability of the CRP from each of these organisms is conserved, highlighting the tight interrelationship between CRP structure and function. Indeed, this taxonomic conservation accounts for the high sequence similarity between mouse and human CRP at the level of both nt (75.63%) and aa (69.78%; see Appendix A, Table A, page 145).

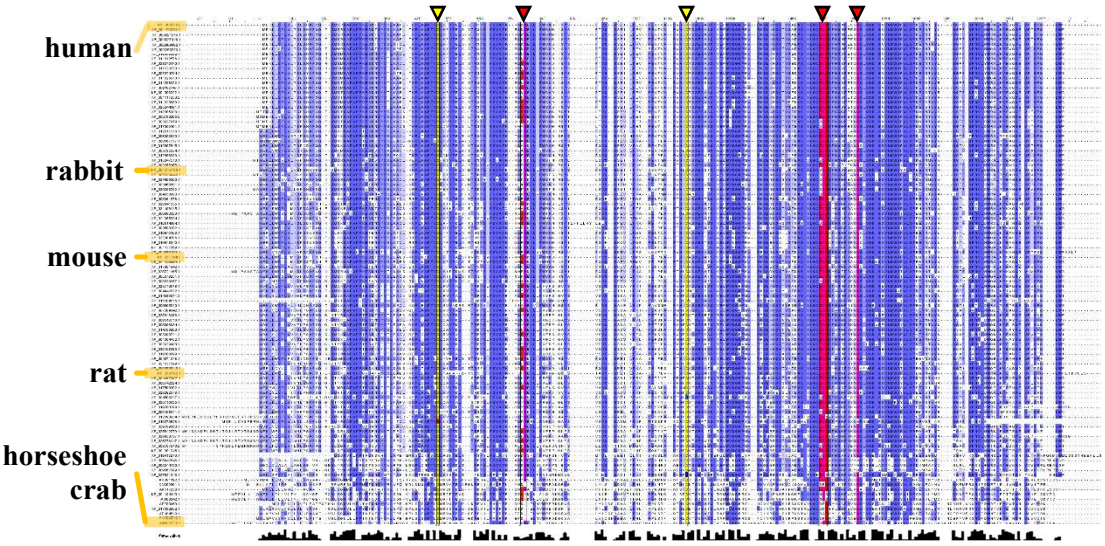



**Figure 8. Multiple sequence alignment of 104 CRP ortholog protein sequences.** Orthologs were calculated by the NCBI eukaryotic genome annotation pipeline and manually selected based on phylogeny comparisons in previous publications. In some cases, more than one isoform:gene exists for a species but only one gene was used for alignment. Sequences were aligned using the default settings of ClustalOmega[91, 92] through the JABAWS web service plugin[93] in Jalview version 2.11.0[94]. Human CRP (top row) was selected to sort the sequences by pairwise identity as calculated by the ClustalOmega alignment. The saturation of blue indicates a higher percent identity between sequences. The conservation across the alignment (bottom black bars) was calculated by AACon[95]. The yellow highlighted residues are the conserved cystines involved in the intrasubunit disulfide bond. The red and pink highlighted residues are involved in $Ca^{2+}$ binding. The highlighted residues are colored based on an identity threshold above 98% (▽) and 25%, 70%, or 98% (left to right ▼, respectively) within the selected position. See Table A in Appendix A for the organisms used ranked by their pairwise identity compared to human CRP based on ClustalOmega alignment.

*Post-Transcriptional Regulation of CRP*

As discussed above, CRP biosynthesis is mainly controlled at the transcriptional level. Briefly, CRP mRNA translation yields a 226 aa peptide containing an 18 aa leader peptide that likely ensures delivery to the ER, where the leader is removed, and the 206 aa peptide is folded into a mature CRP monomer. In the ER, the monomers are assembled into the pentameric CRP protein and a small pool of CRP is maintained. Further control of CRP level at the post-transcriptional stage is known through a few mechanisms, as will be briefly discussed. Notably, it was shown in both healthy human and those under inflammatory stress (e.g. autoimmunity patients or patients with bacterial infection) that

radioiodine labeled CRP ($^{125}$I-CRP) in the serum had an equal half-life (19 h)[96]. These data, along with the mechanisms discussed below, indicate CRP biosynthesis by transcriptional control is the predominant determinant of CRP blood levels.

*mRNA stability*. After transcription ceases CRP mRNA rapidly degrades[97]. CRP mRNA encodes a long 3'UTR that is AU rich (51.2%) and a poly(A) signal found distal to the end of exon 2 (Figure 9). The importance of these genomic features in regulating CRP expression was solidified in the study by Kim et al. wherein they investigated the role of the RNA-binding protein Human antigen R (HuR) and mircoRNAs in post-transcriptional control of CRP expression. HuR is known to bind mRNAs with AU rich regions in their 3'UTR and microRNAs bind complementary sequences in target mRNAs[98, 99]. Indeed, HuR was found to bind two regions in the CRP mRNA 3'UTR and the microRNA 637 bound within these HuR sites (Figure 9)[100].

Additionally, HuR binding was increased following stimulation with IL-6. These findings were supported in a small human cohort (n = 15/group) wherein individuals with high serum CRP (≥ 20 μg/ml) had increased levels of HuR (in their PBMCs) and serum IL-6 compared to the low CRP group (≤ 3 μg/ml). The role of HuR binding the 3'UTR of CRP mRNA both without and with IL-6 stimulation suggests that CRP mRNA is regulated to affect CRP biosynthesis at baseline and during an APR.

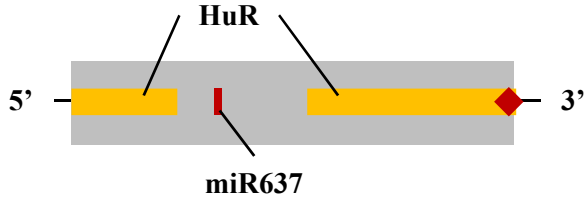



**Figure 9. HuR and miRNA637 binding sites along the 3'UTR of CRP mRNA.** The CRP mRNA 3'UTR (gray bar) is bound along two regions by HuR (yellow bars) and intervening is a small site where the microRNA 637 (miR-637; red bar) binds competitively; poly(A) signal (◆).

*Secretion through the ER.* In the ER, the monomers (sans leader peptide) are assembled into the homopentameric CRP and a small, intracellular pool of CRP is maintained. Investigation into the kinetics of APR-induced CRP biosynthesis revealed that over the course of an APR, CRP secretion from hepatocytes is faster than at baseline[101, 102]. Subsequent studies showed that CRP is retained in the ER by two carboxylesterases, gp60a and gp60b which regulate CRP secretion through two mechanisms. First, during an APR the presence of gp60a and gp60b in the ER falls by two-fold each and second, their affinity for CRP falls by 5- and 25-fold, respectively[103, 104]. Following APR induction, the secretion time for CRP drastically decreases from 18 to 1.25 h and maximum blood levels can occur as quickly as 4 to 6 h after stimulus [101]. Thus, gp60a and gp60b control CRP expression at baseline and upon APR induction their decreased binding of CRP allows for faster release from the ER thereby providing rapid hepatocyte secretion of CRP into the circulation.

*APR-resolution mechanisms.* The acute phase response, as defined in this dissertation, is a rapid (≤ 24 h) liver response to inflammatory insult and the CRP blood level is highly reflective: rapidly rising in serum concentration concordant with the magnitude and duration of the process. Most robustly, CRP expression is largely maintained and amplified by transcriptional control, as discussed above. Additionally, CRP blood levels fall off almost as dramatically as it increases. Although there is no clearly defined mechanism, there is indirect evidence of how CRP levels are brought to baseline. The popular hypothesis is that the resolution of the APR-triggering inflammation leads to decreased stimulation for CRP expression, similarly to other APP. In other words, it is predominately the simple loss of APR-inducing cytokines (e.g. IL-6, IL-1β) to drive *CRP*

transcription. Supporting this hypothesis are a few studies that found that IL-6 induced hepatocyte CRP expression is decreased during IL-4 co-exposure[105, 106].

*In vivo* IL-4 expression likely occurs at the resolution of the APR, thus providing a negative feedback on IL-6 driven responses (i.e. CRP expression). In addition, there are several negative feedback mechanisms for the APR cytokines *per se*, such as the IL-1 receptor antagonist and IL-6 receptor shedding, which would prevent further cytokine-induced CRP expression[41]. On a transcriptional level, the transcription factor OCT-1 competes with C/EBPβ and NF-κB binding (red box, Figure 5), resulting in their displacement during IL-6 and IL-1β exposure[107]. Thus, OCT-1 somehow in involved in down-regulating *CRP* expression following APR induction.

Beyond this there is little understanding of how CRP expression decreases following an APR. While we know that the half-life of CRP in the serum is 19 h[108], how or if CRP is degraded or cleared from circulation is still unknown. We previously showed that mice injected intravenously with exogenous human CRP maintained the blood CRP levels for 24 h, even with a bilateral nephrectomy (i.e. in the absence of renal clearance/filtering of the blood)[109]. This suggests that CRP in the circulation is cleared by the kidney, yet no CRP is found in the urine of CRPtg undergoing an APR.

*CRP Receptors*

Early on it was recognized that CRP influenced the activities and functions of various leukocytes[23], including myeloid cells[110-112], B cells[113], and T cells[114]. While CRP can influence a wide array of cell types, most studies of its biology have focused on myeloid cells. Furthermore, how CRP enacts its cellular effects is primarily through

engagement of Fcγ receptors (FcγR)[33, 85] but there is evidence that CRP also interacts with FcαRI[115] and LOX-1[34, 116].

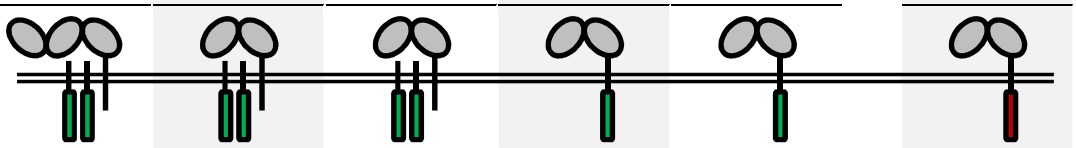
Fcγ receptors are prototypically known as receptors for the Fc region of IgG. However, FcγRs have important roles in mediating myeloid cell biology, including on dendritic cells, monocytes/macrophages, and neutrophils[117, 118]. Once bound by ligand, FcγR signaling cascades are mediated through either an immunoreceptor tyrosine-based activation motif (ITAM) or an immunoreceptor tyrosine-based inhibitory motif (ITIM) and are thus classified as activating or inhibitory (Table 1). Activating FcγR signaling is mediated either through an associated common gamma chain bearing two ITAM domains (FcγRI, FcγRIII, and FcγRIV) or directly through an ITAM on the cytoplasmic tail (FcγRIIA and FcγRIIC). Notably, both in humans and in mice, FcγRIIB is the only inhibitory FcγR (note the ITIM on its cytoplasmic tail, red box, Table 1). FcγRIIB potently inhibits the signaling cascades of ITAM-bearing FcγRs (particularly FcγRI), however FcγRIIB is a low-affinity receptor.

CRP binds to FcγRs with a 1:1 stoichiometry. On the A face, each monomer contains a furrow, partially formed by the α-helix, that is tilted slightly inward towards the central pore. The cleft from two opposing monomers coordinates CRP to dock, A face down, across two immunoglobulin domains of the FcγR (gray ovals, Table 1) that are facing upwards from the cell membrane [85]. It is postulated that this configuration allows for CRP to additionally bind ligand with the B face and act as an adapter molecule.

The receptor that most potently signals CRP effects is FcγRIIB (CD32B)[119], notably through its ability to mediate phagocytosis by human granulocytes and monocytes[119-121]. Because of the evolutionary conservation of CRP and FcγR structures,

human CRP can utilize mouse FcγRs[122]. Indeed, in CRPtg mice where mouse FcγRIIB was replaced with human FcγRIIB, CRP protected against experimental autoimmune encephalomyelitis similar to what was seen in CRPtg mice expressing mouse FcγRIIB[123]. Other studies have shown the importance of FcγRIIB in realizing CRP biology *in vivo*[124, 125]. CRP binds FcγRs with a higher dissociation constant than IgG1[85].

**Table 1. FcγR expression in human (H) and mouse (M) with respect to CRP binding (*).**

| activating | | | | | inhibitory |
|---|---|---|---|---|---|
| **FcγRI** | **FcγRIII** | **FcγRIV** | **FcγRIIA** | **FcγRIIC** | **FcγRIIB** |
| CD64 | CD16 | CD16.2 | CD32A | CD32C | CD32B |
|  | | | | | |
| H | H | H | H | H | H |
| M | M | M | | | M |
| ** | * | | ** | | *** |

## Dissertation Objective

This dissertation project sought to further the understanding of CRP biology and alleviate the gap between basic and clinical research[126]. Starting at the bedside, high CRP was associated with every stage of acute kidney injury (AKI) and eventual patient mortality. To test whether CRP had any causal role in AKI pathogenesis we utilized the established, robust, and stalwart human CRP transgenic mice (CRPtg)[36] and CRP knockout mice ($CRP^{-/-}$)[127] in a surgical pre-clinical model of AKI (bilateral renal ischemia/reperfusion injury). When subjected to bilateral renal ischemia/reperfusion injury (IRI) the CRPtg mice had worse outcomes compared to wild type and $CRP^{-/-}$ mice. Specifically, the CRPtg mice had severe physical damage to the kidneys (disruption of the tubular architecture), increased urine albumin and serum creatinine, and increased infiltration of myeloid derived cells with suppressor functions (MDSC), specifically polymorphonuclear MDSC (PMN-MDSC). Thus, I sought to determine whether human CRP i) influenced MDSC generation and their function, and ii) what effect MDSC have on the health of renal epithelia, a cell type targeted by renal IRI damage.

Throughout the course of this project I saw increasing evidence that CRP instructs a suppressive/regulatory phenotype as myeloid cells are generated from bone marrow progenitors. Throughout the extensive amount of CRP research, only a few reports have studied this effect of CRP and were conducted nearly 40 years ago. I elaborate on this thesis in more detail at the end in the *Discussion*, where I tie these earlier observations to my own and to the objective stated above. In light of this thesis I have developed, I further explore the future of CRP biology research in the cancer setting where again the discrepancy between clinical correlations need to be separated from causality.

## Acute Kidney Injury

Acute kidney injury (AKI) occurs in the community[128], during hospital admittance[129], and most especially in the intensive care unit[130] and its rate across the globe is increasing[131, 132]. AKI is the rapid loss of renal function that is commonly diagnosed and staged using the definitions of the kidney disease: improving global outcomes initiative (KDIGO) established in August 2012[133]. The KDIGO criterion combined and simplified the earlier classification criterion[134, 135]. As a result more patients are staged: those with milder forms of kidney function loss that later have disproportionately high adverse outcomes. For the purposes of this dissertation KDIGO will be the criteria for further discussion of AKI based on its high use in the clinic and research publications. The KDIGO diagnoses AKI based upon the following criteria and its severity staged (Table 2).

- Increase in serum creatinine by ≥0.3 mg/dL (≥26.5 μmol/L) ≤48 h, or
- Increase in serum creatinine to ≥1.5 times baseline,
  which is known or presumed to have occurred within the prior 7 d, or
- Urine volume <0.5 mL/kg/h for 6 h

**Table 2. KDIGO criteria for staging AKI severity.**

| Stage | Serum creatinine | Urine output |
|---|---|---|
| 1 | • 1.5 – 1.9 times baseline, or<br>• ≥0.3 mg/dL (≥26.5 μmol/L) | • <0.5 mL/kg/h for 6 – 12 h |
| 2 | • 2.0 – 2.9 times baseline | • <0.5 mL/kg/h for ≥12 h |
| 3 | • 3.0 times baseline, or<br>• ≥4.0 mg/dL (≥353.6 μmol/L), or<br>• Initiation of renal replacement therapy, or<br>• If <18 y/o, eGFR <35 mL/min/1.73 $m^2$ | • <0.3 mL/kg/h for ≥24 h, or<br>• Anuria for ≥12 h |

KDIGO recommends patient classification that results in the highest stage of injury.
eGFR: estimated glomerular filtration rate

Hospitalizations with AKI are increasing and occur at a higher rate in females, people aged 65+ (Figure 10), and in patients with comorbidities. The healthcare of AKI does not cede upon hospital discharge: in 2016 the probability of hospital re-admittance for AKI ranged from 21 – 51% (Figure 10) and in 2014 – 2015 there was a 30.8 – 33.8% likelihood of developing chronic kidney disease (CKD) or end-stage renal disease[129]. These poor prognoses are likely due to AKI treatment restricted to alleviating symptoms; in severe cases renal replacement therapy (dialysis); eventual kidney transplant is a last resource despite the improvement in donor:recipient matching[136, 137] and survival of patient and their graft with the ever-still wide gap between available kidneys and needed transplants. The best and current therapeutic focus is prevention of AKI. The gap in therapies can be attributed to the broadly encompassing KDIGO classification that envelops the various etiologies of AKI: pre-renal, renal, and post-renal. Each create differing cascades of pathophysiology yet have an underlying and unifying process is inflammation.

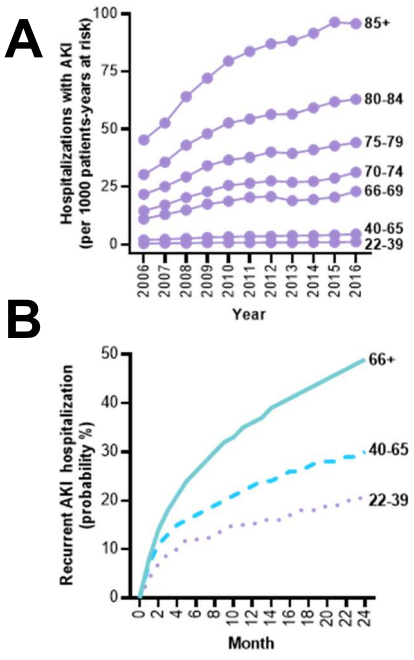



**Figure 10. AKI hospitalizations by age group.** (A) The unadjusted rate of hospitalizations with AKI over time and (B) the probability of an AKI readmission to the hospital by age group within 2 y of hospital discharge. Patient data is from the special analyses, Medicare 5% sample and Optum Clinformatics™. Patient age determined on 1 January for the date shown. Medicare patients were aged 66+ who had both Medicare Parts A & B, no Medicare Advantage plan, no ESRD by first service date from Medical Evidence form, and were alive on 1 January for the date shown. Optum Clinformatics™ enrolled commercial insurance patients were aged 22 - 65, with no ESRD diagnosis, and were alive on 1 January for the date shown. These data were supplied by the United States Renal Data System (USRDS) in their most recent publication in 2018. The interpretation and reporting of these data are the responsibility of R. Jimenez and in no way should be seen as an official policy or interpretation of the U.S. government.

The clinical definition of inflammaiton in AKI has not been described and is predominately due to the acute nature of the disease and inability to access and study patient biopsies in an ethical manner (i.e. invasively sample). Unsurprisingly, CRP is associated with every stage of AKI progression (Figure 11).The current knowledge of CRP or any inflammatory process during AKI is gleaned from animal models. A preclinical model of AKI that ours and other laboratories use is a surgical bilateral renal ischemia/reperfusion (I/R) injury model, which mimics the AKI experienced following myocardial infarction or when a donor kidney is removed for transplantaion.

In I/R-induced AKI the initial hypoxic environment leads to acute tubular necrosis, obstruction of collecting ducts and/or renal vasculature, and inflammation-mediated immune responses. In this I/R model, the early proximal tubule cells are particularly vulnerable to the hypoxia-induced cell death, leading to loss of their brush border and disruption of the epithelial basement membrane. As a consequence of their demise and the now-porous tubule, the filtrate back leaks. The sloughing of dead and viable cells leads to cast formation[138]. Furthermore, we know that there is activation of

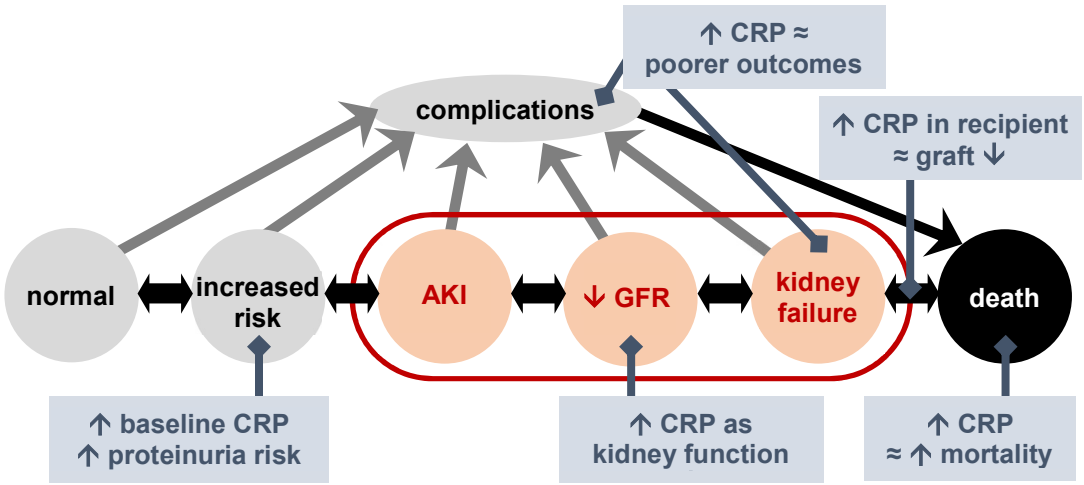



**Figure 11. Human CRP blood level is associated with all stages of AKI.** As AKI progresses (left to right) there is coincident inflammation that associates with CRP. The '≈' indicates a correlation. Implications were drawn from the following references.

complement pathways, production of cytokines and chemokines, and induction of the acute phase response[139]. Ultimately, the combination of compromised renal epithelial and endothelial architecture, dead cells, debris, and the influx of leukocytes can culminate in renal blood flow and filtrate obstruction[140]. Together with persistent inflammatory signals and impaired renal stromal cell regeneration results in ineffective resolution of the injury. As a consequence, patients often develop chronic kidney disease, then on to end-stage renal disease, and eventual death.

Early after AKI myeloid cell involvement begins with the activation of renal resident myeloid cells, macrophages and dendritic cells (DC). These cells are believed to add to the cytokine and chemokine production to draw in more leukocytes[139]. Depletion and adoptive transfer of macrophages before renal I/R showed that these cells both promote the injury but become critical for repair later in AKI sequelae[141]. I argue that this macrophage population likely includes the monocytic subtype of myeloid derived cells with suppressor phenotypes (M-MDSC). DCs appear to turn on turn on T cell responses towards the repair phase but this action does not appear to be important[142]. Neutrophils detrimentally contribute to kidney I/R, in that they damage the renal epithelial and endothelial cells (increasing permeability of the blood flow and the filtrate into the interstitium) by the production of proteases, myeloperoxidase, reactive oxygen species (ROS), and even cytokines[143, 144]. I propose that within this neutrophil population is the granulocytic subtype of MDSCs (G-MDSC) that in CRPtg largely accounts for their heightened kidney damage following I/R.

Myelopoiesis and Development of Myeloid Suppressor Cells

The cellular arm of innate immunity is largely carried out by myeloid cells that act as sentinels while in tissues and in the blood circulation. Differentiated myeloid cells have a low self-renewal and proliferation capacity in comparison to their common myeloid progenitor (cMP) and must be replaced. During the steady state, hematopoiesis in the bone marrow (BM) can be organized into a hierarchical structure (Figure 12). In emergency settings however, hematopoiesis shifts to an almost exclusive production of myeloid cells, especially granulocytes, e.g. neutrophils. Resolution of the insult driving this emergency myelopoiesis restores the balance of hematopoiesis towards the balance seen at the steady state (for instance, more lymphocyte production). Interestingly, there are growing reports that the scales do not completely return to their earlier set point and instead the BM has a lower threshold for shifting towards myelopoiesis in response to certain stimuli, a form of "innate memory"[145].

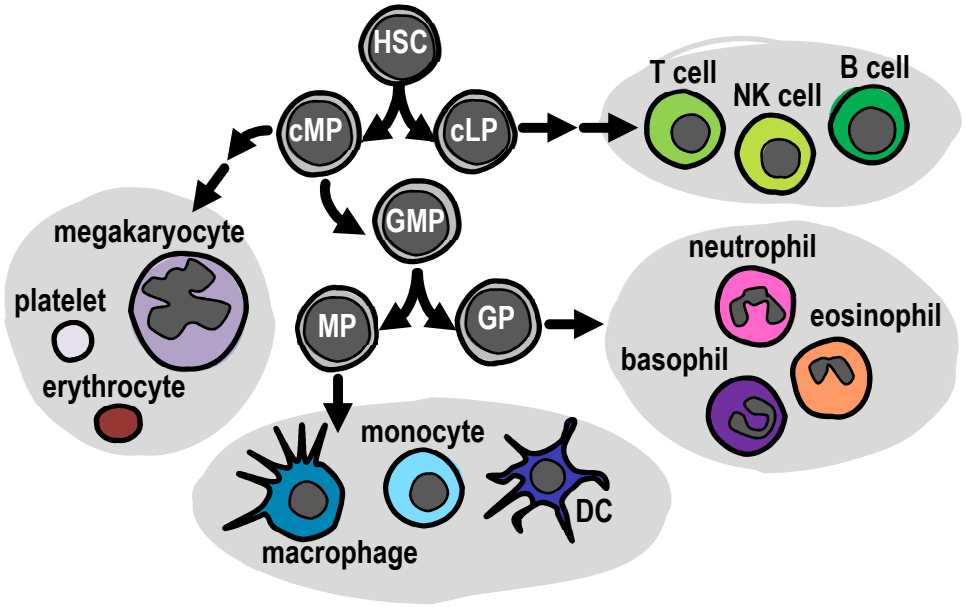



**Figure 12. Steady state hematopoiesis can be organized into a hierarchy of progenitors.** As hematopoiesis proceeds (top to bottom) the proliferation and self-renewal capacity of the progenitors (gray cells) is lost as the cells differentiate. HSC: hematopoietic stem cell; cMP: common myeloid progenitor; cLP: common lymphoid progenitor; GMP: granulocyte-monocyte progenitor; MP: monocyte progenitor; GP: granulocyte progenitor.

Emergency or demand-adapted myelopoiesis can be stimulated by a variety of insults, including infection and sepsis, during the acute phase response, and cancer, occurring over an acute or chronic timeframe. In the case of cancer, it was noted that there is a rise in immature myeloid cells that enter the tumor and immunosuppress anti-tumor responses, especially those of T cells. These cells were termed myeloid derived suppressor cells (MDSC) and over the past decade many reports have established their identity as a unique cell subtype but still debated is whether these cells are indeed immature or merely plastic. There is evidence that those cells that come directly from the BM progenitor compartment are simply a suppressor or regulatory phenotype of the monocyte or granulocyte lineage. Additionally, MDSCs can arise from reprogrammed "mature" monocytes and granulocytes by pathological stimuli in the periphery. In either case of their ontogeny, MDSCs can be subtyped into monocytic (M-) or granulocytic (G-) MDSCs and by flow cytometry appear to have similar lineage surface phenotypes to their differentiated/mature myeloid counterparts yet retain an immature surface phenotype in regard to activation markers. In humans, MDSCs are largely studied from tumor biopsies and select tissues as well as from the peripheral blood monocyte cell (PBMC) layer (i.e. low-density) or potentiated from human blood leukocytes[146]. While there are differences in marker expression between human and mouse (Table 3), MDSCs have been studied extensively in the mouse.

**Table 3. Mouse and human MDSC surface phenotype.**

| | Mechanism | Mouse | Human |
|---|---|---|---|
| **M-MDSC** | RNS; ARG | $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{hi}$ | $CD14^{+}$ $HLA\text{-}DR^{-/lo}$ |
| **G-MDSC** | ROS; IDO | $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{lo}$ | $CD11b^{+}$ $CD15^{+}$ $CD14^{-}$ $CD33^{+/lo}$ $CD66b^{+}$ |

RNS: reactive nitrogen species production; ARG: arginase 1 expression;
ROS: reactive oxygen species generation; IDO: indolamine-2,3-dioxygenase
Markers were tabulated from [147].

The term ‘immature’ in G-MDSC subtype can refer to the non-uniform amount of hyper-segmentation morphology of the nucleus seen in ‘mature’ neutrophils. There are differences in notation for these cells, wherein some use PMN-MDSC in homage to their neutrophil/polymorphonuclear cell phenotype yet others use G-MDSC to recognize that within the surface phenotype there are a variety of nuclear morphologies represented[148]. As you read this dissertation, you will notice that there is a mix of G-MDSC and PMN-MDSC usage that is referring to the same population; our nomenclature has evolved alongside the MDSC biology field. Likewise, our definition of ‘MDSC’ has evolved and is explored further in the Discussion section *CRP Instructs a Regulatory Myeloid Cell Phenotype*. In the last two chapters of this dissertation, MDSCs are defined as myeloid derived cells with suppressor functions.

The above distinction is of paramount importance to the field of myeloid cell biology: whatever semantics each laboratory or report uses they absolutely must demonstrate MDSC suppressive function with a functional assay(s)[147, 149]. M-MDSCs and G-MDSCs have differing suppression mechanisms (Table 3) that is akin to their differentiated myeloid counterparts with the distinction that MDSCs aberrantly and pathologically suppress the immune reaction[148]. Note that MDSCs suppress immune responses without the direction of antigen and instead rely on soluble mediators that are non-specific. For example, any nearby cells susceptible to ROS or RNS and/or arginine or tryptophan depletion will be affected by MDSCs. Experimentally, MDSC suppression is typically demonstrated by their ability to inhibit the proliferation of T cells driven by either antigen or not[150]. The method our laboratory chose for our MDSC studies was non-

specific T cell proliferation in response to stimulation by anti-CD3ε and anti-CD28 antibodies, which is consistent with the field standards produces less artefactual data[151].

MDSCs are proposed to be an evolutionarily conserved phenotype that arose as a normal physiological response to acute reactions and severe inflammatory insults. Indeed, in settings where immunosuppression is warranted for host health, MDSCs are ideally equipped. As such, the characterization of MDSCs as either pro-inflammatory or anti-inflammatory is context dependent, much like CRP. In this dissertation, I assert that CRP is a soluble molecule capable of programming myeloid lineage cells to become MDSC in their phenotype and function during their myelopoiesis from the BM progenitors.

# C-REACTIVE PROTEIN IMPAIRS DENDRITIC CELL DEVELOPMENT, MATURATION, AND FUNCTION: IMPLICATIONS FOR PERIPHERAL TOLERANCE

by

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Abstract

C-reactive protein (CRP) is the prototypical acute phase reactant, increasing in blood concentration rapidly and several-fold in response to inflammation. Recent evidence indicates that CRP has an important physiological role even at low, baseline levels, or in the absence of overt inflammation. For example, we have shown that human CRP inhibits the progression of experimental autoimmune encephalomyelitis (EAE) in CRP transgenic mice by shifting CD4+ T cells away from the TH1 and toward the TH2 subset. Notably, this action required the inhibitory Fcγ receptor IIB (FcγRIIB), but did not require high levels of human CRP. Herein, we sought to determine if CRP's influence in EAE might be explained by CRP acting on dendritic cells (DC; antigen presenting cells known to express FcγRIIB). We found that CRP (50 μg/ml) reduced the yield of $CD11c^{+}$ bone marrow-derived DCs (BMDCs) and CRP (≥5 μg/ml) prevented their full expression of major histocompatibility complex class II and the co-stimulatory molecules CD86 and CD40. CRP also decreased the ability of BMDCs to stimulate antigen-driven proliferation of T cells *in vitro*. Importantly, if the BMDCs were genetically deficient in mouse FcγRIIB then (i) the ability of CRP to alter BMDC surface phenotype and impair T cell proliferation was ablated and (ii) CD11c-driven expression of a human *FCGR2B* transgene rescued the CRP effect. Lastly, the protective influence of CRP in EAE was fully restored in mice with CD11c-driven human FcγRIIB expression. These findings add to the growing evidence that CRP has important biological effects even in the absence of an acute phase response, i.e., CRP acts as a tonic suppressor of the adaptive immune system. The ability of CRP to suppress development, maturation, and function of DCs implicates CRP in the maintenance of peripheral T cell tolerance.

## Introduction

Inflammation is a normal local response to tissue injury and infection. If the insult is sufficiently strong there will follow a systemic response, termed the acute phase response (APR), during which leukocytes release inflammatory mediators (primarily IL-6, IL-1, and/or TNFα) into the circulation that sequentially propel a diversity of effects. During the APR, the liver increases the synthesis of a number of pattern recognition proteins. Among these C-reactive protein (CRP) is the prototype; it is maintained at low levels in normal sera (1–5 μg/ml) (1), but can reach upwards of ~500 μg/ml during inflammation (2). CRP's ability to activate complement, opsonize microbes, bind to phosphatidylserine on apoptotic cells, and bind Fc receptors is well known (2–4) and these biological actions have been studied extensively in the context of CRP's upregulation during inflammation. Increasing evidence indicates that CRP also exerts important biological influences even when its levels remain low as in healthy individuals and when it is only slightly raised as in aging individuals (4).

Previously, we have shown that human CRP transgenic mice (CRPtg) are resistant to experimental autoimmune encephalomyelitis (EAE), a disease comparable to human multiple sclerosis (MS) i.e., they have delayed onset of disease and milder clinical symptoms compared to wild type (WT) mice. Notably, despite the ability of CRPtg to mount a robust human CRP acute phase response, this protection does not require high levels of human CRP. We initially attributed CRP's protective action in EAE to inhibition of encephalitogenic T cells, since *in vitro* CRP reduced T cell proliferation and shifted their cytokine production toward a less noxious $T_H2$ profile (5). Our subsequent studies demonstrated that FcγRIIB$^{-/-}$ mice, which lack expression of this inhibitory receptor, were refractory to CRP's protective action (6), but we did not identify the

FcγRIIB-expressing cell(s) that CRP relied upon. Herein, we demonstrate that CRP impairs the development of bone marrow (BM) cells into $CD11c^{+}$ dendritic cells (DCs), professional antigen presenting cells that express ample FcγRIIB (7), are paramount for robust T cell responses (8), and are known to contribute to EAE/MS (9, 10, 11).

At doses as low as 5 μg/ml, CRP significantly prohibited bone marrow-derived DCs (BMDC) activation/maturation in response to stimulation with lipopolysaccharide (LPS), and impaired the ability of BMDCs to promote antigen-specific T cell proliferation. These suppressive actions of CRP were not evident using $FcγRIIB^{-/-}$ BMDCs, but BMDCs from $FcγRIIB^{-/-}$ mice genetically reconstituted to express a CD11c-driven human FcγRIIB transgene ($_{cd11c}FcγRIIB^{hu}$) were responsive to CRP, i.e., CRP prohibited their activation/maturation in response to LPS and suppressed their ability to promote T cell proliferation. As we previously reported, CRPtg were more resistant to EAE compared to WT, whereas CRPtg lacking expression of endogenous FcγRIIB ($FcγRIIB^{-/-}$/CRPtg), were not. For the latter, however, expression of the CD11c-specific human FcγRIIB transgene fully reconstituted human CRP-mediated protection from EAE.

Based on these new findings, we propose that CRP acts as an endogenous down-regulator of DC development and activation/ maturation, thereby acting as a brake on T cell mediated immunity and shifting the balance toward tolerance. Given that many of the effects of CRP on DCs were observed using ≤10 μg/ml, it is likely that even modest elevation of blood CRP—like that associated with aging (12)—is sufficient to significantly affect T cell tolerance.

## Materials and Methods

### *Mice*

Our human CRPtg have been fully described elsewhere (13, 14). In brief, CRPtg (C57BL/6 background) carry a 31-kb human DNA fragment encoding the *CRP* gene and all the *cis*-acting elements required for tissue specificity and acute phase inducibility, while the *trans*-acting factors required for its human-like pattern of regulation are conserved from mouse to man (13, 14). Consequently, unlike WT, CRPtg exhibit a robust human CRP acute phase response during inflammation. FcγRIIB deficient mice (FcγRIIB$^{-/-}$; B6.129S4-*Fcgr2b*$^{tm1TtK}$ N12) (15) were purchased from Taconic Farms (Germantown, NY). 2D2 mice [C57BL/ 6-Tg(Tcra 2D2, Tcrb 2D2) 1Kuch/J] (16) are transgenic for a T cell receptor (TCR) that recognizes residues 35–55 of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) and were purchased from Jackson Laboratories (Bar Harbor, ME, USA; JAX 006912). OT-II mice [B6.Cg-Tg(Tcra Tcrb)425Cbn/J] (17) are transgenic for a TCR that recognizes residues 323–339 of ovalbumin ($OVA_{323–339}$) and were purchased from Jackson Laboratories (Bar Harbor, ME, USA; JAX 004194). FcγRIIB$^{-/-}$ mice expressing a human *FCGR2B* transgene driven by a mouse CD11c minimal promoter ($_{cd11c}$FcγRIIB$^{hu}$) were generated herein and are fully described in the Section "Results." To date, no embryonic lethality or unusual phenotype has been observed for $_{cd11c}$FcγRIIB$^{hu}$. C57BL/6 mice (WT) were obtained from the Jackson Laboratories (Bar Harbor, ME, USA; JAX 000664). All mice were housed in the same vivarium at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12-h light cycle (6:00 a.m. to 6:00 p.m.), and maintained *ad libitum* on sterile water and regular chow (Harlan Teklad). Mice were 8–12 weeks old when used and both sexes were combined unless specifically noted. All animal use protocols were approved by the

Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and were consistent with the *Guide for the Care and Use of Laboratory Animals; Eighth Edition* (NIH Academies Press, 2011).

*BMDC Cultures*

Bone marrow progenitors were grown under conditions known to drive DC generation and expansion (18, 19). Briefly, BM was harvested from femurs, the red blood cells lysed (Hybri-Max Red Blood Cell Lysing Buffer; Sigma, Salem, MA, USA), and the marrow passed through a 70 μM cell strainer and brought to single-cell suspension in RPMI 1640 (Gibco, Grand Island, NY) containing 5% fetal bovine serum (Gibco), 1% Penicillin/ Streptomycin (Gibco), 2 mM GlutaMAX™ (Invitrogen), non-essential amino acids (Gibco), 55 μM β-mercaptoethanol (Gibco), and 20 ng/ml granulocyte macrophage-colony stimulating factor (Shenandoah Biotechnology, Warwick, PA, USA). BM progenitors were then added to 12-well tissue culture plates ($1 \times 10^6$ cells in 1 ml per well) that were incubated at 37°C, 5% $CO_2$ for 7 days. The culture medium was replaced on days 3 and 5. On day 5, cells were exposed to 50 μg/ml of highly purified human CRP (endotoxin and azide-free CRP from US Biological; Salem, MA, USA), purified chicken $OVA_{323–339}$ peptide (MISC- 011; CPC Scientific, San Jose, CA, USA), or purified $MOG_{35–55}$ peptide (12668-01; Biosynthesis Inc., Lewisville, TX, USA). $OVA_{323–339}$ and $MOG_{35–55}$ loaded BMDCs were subsequently used in BMDC:T cell co-cultures with OT-II and 2D2 T cells, respectively, as described below. To trigger BMDC maturation in some experiments LPS from *Escherichia coli,* serotype 055:B5 (Sigma Aldrich) was added (1 μg/ml) on day 6. Alternatively, culture medium was supplemented with 100 ng/ml interleukin-4 (IL-4; Shenandoah Biotechnology, Warwick, PA, USA). IL-10 and

IL-12p70 production was assessed by ELISA (88-7105-22 and 88-7121-22; Invitrogen, Eugene, OR, USA) according to the manufacturer protocol. Flow cytometry was performed on a BD LSR-II cytometer (described below) and, after excluding dead cells and aggregated cells, BMDCs were identified as $CD11b^+$ $CD11c^+$ cells. For detailed analysis of cell death, cells were stained with Annexin V and 7-AAD and were defined as early apoptotic (Annexin $V^+$ 7-$AAD^-$), late apoptotic (Annexin $V^+$ 7-$AAD^+$), necrotic (Annexin $V^-$ 7-$AAD^+$), or live (Annexin $V^-$ 7-$AAD^-$).

*T Cells And BMDC:T Cell Co-Cultures*

From OT-II and 2D2 mice, the spleens and lymph nodes (axillary, brachial, inguinal) were harvested and mechanically homogenized, the red blood cells lysed, and the homogenate passed through a 70 μM cell strainer, and brought to single-cell suspension in media at $1 \times 10^8$ cells/ml. $CD4^+$ T cells were enriched by negative selection according to the manufacturer's guidelines using a kit from StemCell Technologies (Vancouver, BC, Canada). Enriched $CD4^+$ T cells were then stained for 20 min with 1 μM CellTrace carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Eugene, OR, USA). BMDCs, cultured as described above, were treated with $MOG_{35–55}$ or $OVA_{323–339}$ peptide on day 6. On day 7, the peptide-loaded BMDCs were mixed with the freshly isolated and CFSE-stained $CD4^+$ T cells (1:5 ratio in triplicate), placed into 96-well round bottom plates, and incubated for 3 days before analysis of $CD4^+$ T cell proliferation (dilution of CFSE). BMDC:T cell co-cultures exposed to plate-bound anti-CD3ε and soluble anti-CD28 antibodies (both from Biolegend, San Diego, CA, USA) served as positive controls

*Antibodies and Flow Cytometry*

Cells were washed with PBS, spun down at 300 × *g* for 5 min at 4°C, stained with the viability dye eFluor 780 (eBioscience, San Diego, CA, USA) for 30 min at room temperature, fixed in Fixation Buffer (Biolegend, San Diego, CA, USA) for 10 min at room temperature, blocked with anti-mouse CD16/32 (clone 93; eBioscience) at 4°C for 15 min, and stained with specific antibodies at 4°C for 30 min. For BMDCs, we used anti-mouse CD11c (clone N418), MHC class II IA/IE (clone M5/114.15.2), CD40 (clone HM40-3), CD80 (clone 16–10 A1), CD86 (clone GL-1) (all from Biolegend), and FcγRIIB (clone AT 130-5, Bio Rad, Hercules, CA, USA), and anti-human FcγRIIB (clone AT 10, AbD Serotec, Raleigh, NC, USA). For T cells we used anti-mouse CD4 (clone RM4-5) (Biolegend). Stained and labeled cells were run on a BD LSR-II cytometer and the acquired data analyzed using BD FACSDiva version 6.1.3 and FlowJo version 10.3. For all gating analyses, debris was gated out using a FSC by SSC dot plot, followed by selection of single cells using a SSC-A by SSC-H dot plot, and live cells were selected based on the viability dye eFluor 780 dot plot. For assessment of T cell proliferation (CFSE dilution), the bounds for the $CD4^+$ $CFSE^+$ "parents" gate was determined using unstimulated T cells and the bounds for the "progeny peaks" were based on anti-CD3ε/anti-CD28 stimulated T cells (see Figure 3A). As T cells divide, the progeny:parent ratio increases.

*Experimental Autoimmune Encephalomyelitis*

Experimental autoimmune encephalomyelitis was induced as we described previously (5, 6, 20). Briefly, 10–12-week-old mice were immunized subcutaneously with 150 μg MOG35–55 emulsified in Freund's complete adjuvant plus 400 μg heat-

killed *Mycobacterium tuberculosis* (Difco, Detroit, MI, USA). On days 0 and 2, mice received an intraperitoneal injection of 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA). For 30 days thereafter the development of EAE was monitored daily. EAE symptoms were scored on a clinical scale ranging from 0 to 6 as follows: 0, asymptomatic; 1, loss of tail tone; 2, flaccid tail; 3, incomplete paralysis of one or two hind limbs; 4, complete hind limb paralysis; 5, moribund (at which case the mouse was humanely euthanized); 6, dead. For mice that developed EAE, the day of onset was defined as the first of two consecutive days, wherein the clinical score was ≥2.

*Statistical Analysis*

Raw data were pooled and are expressed graphically as the mean ± SEM or SD, as noted. Group comparisons were done using one-way analysis of variance (*ANOVA*) followed by *post hoc* Bonferroni's and Tukey's multiple comparison tests, or using linear trends tests. Differences were considered significant when $p$ was $<0.05$. For EAE, the maximum clinical score achieved by each animal during the 30-day observation period was used to calculate the average maximum clinical score (a measure of severity). To study the time-course of disease, average clinical scores were calculated and plotted daily for each group of mice, and cumulative disease index (CDI) was calculated by area under the curve analysis. Statistical analyses were done using GraphPad Prism version 7.00.

## Results

*CRP Suppresses Generation and Maturation of BMDCs*

We first examined the influence of human CRP on the generation of DCs from BM progenitors. On day 7 of culture, nearly 90% of all cells were viable (dashed horizontal lines in Figure 1A) and the cultures routinely achieved a yield of nearly 75% BMDCs (dashed horizontal line in Figure 1B). Whether CRP at 10 or 100 μg/ml was added on day 0 or 6 of culture it had no significant effect on cell viability (Figure 1A). CRP treatment also had no effect on the proportion of early apoptotic, late apoptotic, and necrotic BM cells (data not shown). However, CRP treatment did significantly decrease (by 10–15%) the proportion of $CD11b^+$ $CD11c^+$ BMDCs that developed (Figure 1B). Notably, when CRP was added at the initiation of culture, the inhibitory effect on the final yield of BMDCs was strongest (Figure 1B) and was dose- dependent (Figure 1C). These results show that while CRP has no significant influence on the viability of

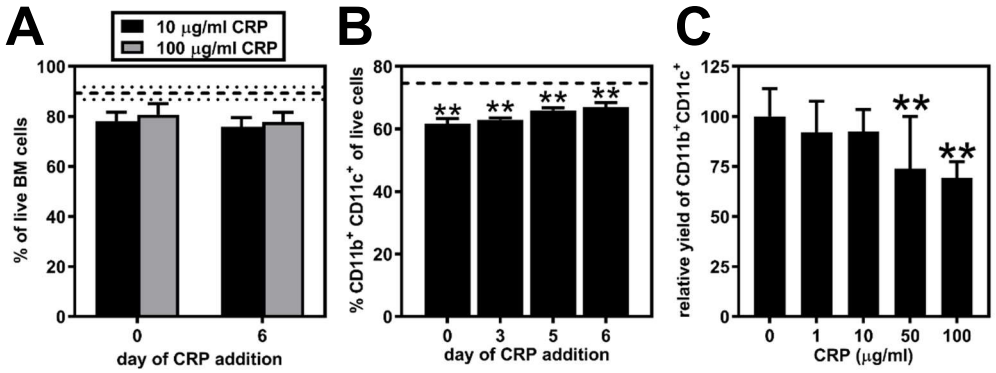


**Figure 1. CRP impedes the generation of $CD11b^+CD11c^+$ BMDCs in a temporal and dose-dependent fashion.**(A) CRP addition to bone marrow cultures on day 0 or on day 6 had no significant effect on cell viability. The horizontal dashed lines indicate cell viability of 89.2% ± 2.52 (mean ± SD) without CRP. (B) The proportion of live cells that were $CD11b^+CD11c^+$ BMDCs was significantly reduced by addition of CRP (50 μg/ml) on the indicated day of culture. The horizontal dashed line indicates the average proportion of BMDCs generated in the absence of CRP (74.6% ± 0.57 SD). (C) The relative yield of $CD11c^+$ BMDCs was reduced in a dose-dependent fashion by CRP (1 – 100 μg/ml) added on day 0 of culture. The symbols indicate the results of one-way ANOVA with Tukey's multiple comparisons tests compared to cultures not treated with CRP, $p < 0.005$ (**) (n = 3 – 9 per group)

cultured BM progenitors, it does significantly impede the generation of $CD11b^+$ $CD11c^+$ BMDCs in both a temporal and dose-dependent manner.

Next, we assessed the influence of CRP on activation/maturation of BMDCs. Treating immature BMDCs with CRP (50 μg/ml) had no effect on their surface expression of MHC class II, CD86, CD40, and CD80 (Figure 2A), whereas treatment of immature BMDCs with LPS (1 μg/ml) significantly upregulated these markers (Figure 2A), indicative of BMDC maturation. While CRP did not trigger BMDC maturation, CRP did significantly inhibit the LPS-triggered increase in surface expression of MHC

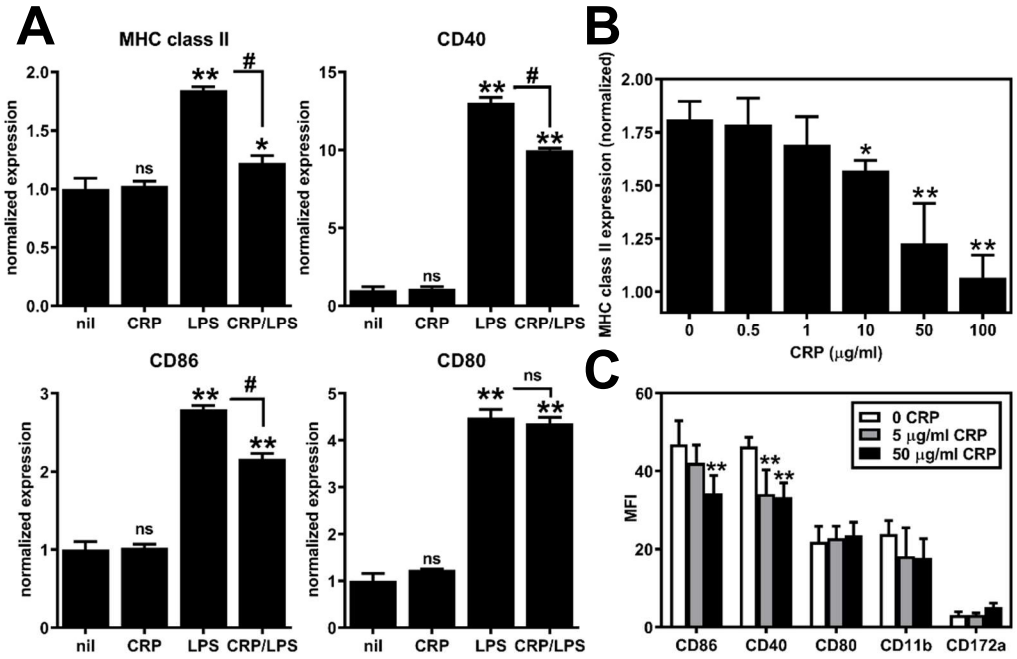


**Figure 2. CRP suppresses expression of MHC class II, CD86, and CD40 on LPS-matured BMDCs in a dose-dependent manner.** (A) Surface expression of MHC class II, CD86, CD40, and CD80 on immature $CD11b^+CD11c^+$ BMDCs left untreated (nil) or treated with CRP (50 μg/ml on day 5), and on BMDCs matured with LPS (1 μg/ml on day 6) or treated with CRP (50 μg/ml on day 5) and LPS (1 μg/ml on day 6) (CRP/LPS). Expression of each marker (MFI of flow cytometry) is normalized to expression on untreated immature BMDCs (nil). The symbols above each bar indicate $p < 0.05$ (*), or $p < 0.005$ (**) compared to "nil." The symbols above each bracket indicate $p < 0.005$ (#) for the LPS versus CRP/LPS groups. One-way ANOVA with Tukey's multiple comparisons tests. (B, C) CRP dose-dependent suppression of expression of MHC class II (top) and CD86 and CD40 (bottom) by LPS-treated BMDCs. MHC class II expression is normalized as in (A). The symbols indicate the results of one-way ANOVA with Tukey's multiple comparisons tests, $p < 0.05$ (*) and $p < 0.005$ (**) compared to no CRP (n = 2 – 6 per group).

class II and the co-stimulatory markers, CD86 and CD40 (Figure 2A). Also, the suppressive effect of CRP on LPS-triggered BMDC maturation was dose-dependent, as evidenced by a stepwise reduction of MHC class II, CD86, and CD40 (Figure 2B). This suppressive effect was specific as CRP had no effect on the expression of CD80, CD11b, or CD172a (Figure 2B). Finally, BMDCs treated with LPS (1 μg/ml) robustly produced both the T cell suppressive cytokine IL-10 and the T cell stimulatory cytokine IL-12p70 (225.7 ± 8.8 and 1245.8 ± 191.0 ng/ml, respectively), but the production of both cytokines was significantly suppressed by CRP (no detectable IL-10 and 773.2 ± 13.2 ng/ml IL-12p70; $p < 0.05$, *t*-tests). These data demonstrate that CRP dose-dependently prohibits LPS-triggered activation/maturation of BMDCs and limits their production of IL-10 and IL-12p70, cytokines with pleiotropic effects in immunoregulation.

*CRP Inhibits BMDC-Mediated Stimulation of Antigen-Specific T Cell Proliferation*

We next sought to determine if the observed effects of CRP on BMDC activation/maturation phenotype and cytokine production affects their T cell stimulatory function. We found that CRP (1–100 μg/ml) had no significant effect on the proliferation of OT-II T cells co-cultured with BMDCs in the absence of any stimulus (Figure 3B; nil) or in the presence of T cell activating antibodies (Figure 3B; CD3/CD28). Importantly however, when BMDCs loaded with $OVA_{323–339}$ peptide were used as APCs, the addition of CRP caused a dose-dependent inhibition of OT-II T cell proliferation (Figure 3B OVA). Using the MOG TCR-transgenic model (2D2) we obtained similar results, i.e., CRP (50 μg/ml) significantly inhibited the proliferation of 2D2 T cells co-cultured with BMDCs loaded with $MOG_{35–55}$ peptide (Figure 3C). These data confirm that CRP's prohibition of BMDC activation/maturation and cytokine production reduces their ability

to stimulate antigen-specific T cell proliferation. The fact that in both model systems, CRP had no effect on T cells directly stimulated with anti-CD3ε/anti-CD28 antibodies shows that CRP's influence on T cell proliferation must be *via* its actions on BMDCs.

*CRP Does Not Prohibit the Activation/Maturation of FcγRIIB−/− BMDCs*

C-reactive protein binds to both activating and inhibitory Fc receptors, thereby triggering a diversity of cellular responses *in vitro* (2, 21) and many of the *in vivo* biological actions of human CRP in CRPtg are fully supported by FcγRIIB (6, 22). Since FcγRs *per se*, and FcγRIIB in particular, are widely expressed by both human and mouse DCs (7), we generated DCs using FcγRIIB$^{-/-}$ BM to test if CRP's influence on BMDC phenotype and function required FcγRIIB. Like the expression on immature WT BMDCs (Figure 2A), expression of MHC class II, CD80, CD40, and CD86 on immature FcγRIIB$^{-/-}$ BMDCs was unaffected by CRP alone (50 μg/ml), and LPS triggered their

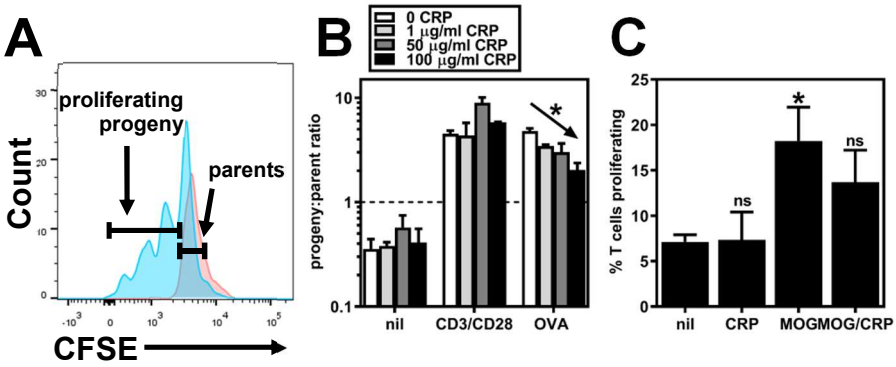


**Figure 3. CRP inhibits BMDC-mediated/antigen-driven T cell proliferation.** (A) Typical flow cytometry histograms for CFSE-labeled OT-II T cells harvested 3 days after co-culture with BMDCs without antigen (parental generation, red) and with BMDCs loaded with $OVA_{323–339}$ peptide (progeny generations, blue). (B) Proliferation of OT-II T cells co-cultured with antigen-naïve BMDCs and no other stimulant (nil), or with anti-CD3ε/ anti-CD28 antibodies (CD3/CD28), and co-cultured with $OVA_{323–339}$ peptide-loaded BMDCs (OVA), without or with addition of CRP. The diagonal arrow indicates $p < 0.0001$ (*) for a linear trend test of column means in left-to-right column order. (C) 2D2 T cell proliferation in the presence of antigen-naïve BMDCs without (nil) or with 50 μg/ml CRP, or in the presence of $MOG_{35–55}$ peptide-loaded BMDCs without (MOG) or with 50 μg/ml CRP (MOG/CRP). The symbols indicate $p < 0.05$ (*) for one-way ANOVA with Tukey's multiple comparisons tests compared to nil (n = 3 – 6 per group).

increase (Figure 4A). However, in stark contrast to its effect on LPS-matured WT BMDCs (Figure 2A), CRP did not impair the LPS-triggered upregulation of MHC class II, CD86, and CD40 by FcγRIIB$^{-/-}$ BMDCs (Figure 4A). Like for WT BMDCs, expression of IL-10 by LPS-treated FcγRIIB$^{-/-}$ BMDCs (308.5 ± 12.5 ng/ml) was lowered by CRP (69.6 ± 8.7 ng/ml). However, unlike for WT BMDCs, for FcγRIIB$^{-/-}$ BMDCs treated with LPS the amount of IL-12p70 produced (948.9 ± 25.3 ng/ml) was not reduced by CRP (1017.1 ± 51.6 ng/ml). These findings strongly suggest that FcγRIIB expression is required for CRP to prohibit LPS-induced activation/ maturation of BMDCs and to suppress production of the T cell stimulatory cytokine IL-12p70. As expected, when $MOG_{33–55}$ peptide- loaded FcγRIIB$^{-/-}$ BMDCs were used as APCs, CRP (50 μg/ml) did not impair their proliferation (Figure 4B). In our hands, FcγRIIB$^{-/-}$ BMDCs did not

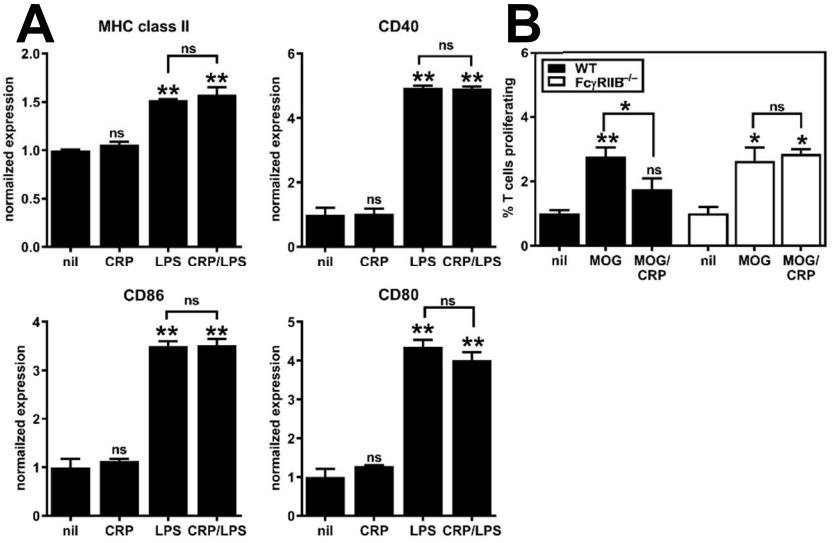


**Figure 4. CRP-mediated prohibition of LPS-induced maturation of BMDCs and inhibition of BMDC-mediated/antigen-driven proliferation of 2D2 T cells is FcγRIIB-dependent.** (A) Surface expression of MHC class II, CD86, CD40, and CD80 on FcγRIIB$^{-/-}$ BMDCs left untreated (nil), or treated with CRP (50 μg/ml), LPS (1 μg/ml), or CRP and LPS. Each marker's expression is normalized to the nil group and the symbols directly above each bar indicate $p < 0.05$ (*), or $p < 0.005$ (**) compared to nil. There was no significant difference between the LPS versus CRP/LPS groups. One-way ANOVA with Tukey's multiple comparisons tests (n = 3 experiments). (B) 2D2 T cell proliferation in the presence of wild type versus FcγRIIB$^{-/-}$BMDCs. BMDCs were antigen-naïve (nil) or $MOG_{35–55}$ peptide-loaded and CRP was at 50 μg/ml. The symbols above the bars indicate $p < 0.05$ (*) and $p < 0.005$ (**). The symbols above the brackets compare the MOG/CRP versus MOG groups. One-way ANOVA with Tukey's multiple comparisons tests (n = 3 per group).

stimulate OT-II T cell proliferation even when loaded with $OVA_{323–339}$ (data not shown), precluding us from assessing if CRP requires FcγRIIB in the OT-II model system. Nevertheless, the results from the 2D2 model confirmed that CRP's ability to prohibit BMDC stimulation of an antigen-specific T cell response is facilitated by FcγRIIB expressed on BMDCs.

*Transgenic Expression of Human FcγRIIB Supports Human CRP's Actions on Mouse FcγRIIB$^{-/-}$ BMDCs*

The apparent requirement of mouse FcγRIIB for human CRP- mediated prohibition of BMDC activation/maturation and 2D2 T cell proliferation prompted us to investigate this biology further. Accordingly, we generated FcγRIIB$^{-/-}$ mice that express a human *FCGR2B* transgene. Expression of the human FcγRIIB receptor was restricted to DCs by using a vector that contains the CD11c minimal promoter (kindly provided by Dr. Thomas Brocker, Institute for Immunology, LMU Munich Goethestr. 31, D-80336 Munich, Germany) (8). Briefly, a full-length cDNA clone encoding human *FCGR2B* (23) was inserted into the vector (Figure 5A) to drive *FCGR2B* expression on $CD11c^+$ DCs in all mouse tissues. Transgenic mice ($_{cd11c}$FcγRIIB$^{hu}$) were then established by injecting the construct directly into fertilized FcγRIIB$^{-/-}$ eggs in the UAB Transgenic & Genetically Engineered Models Core. Offspring were screened for presence of the human transgene by PCR and flow cytometry was used to detect surface expression of human FcγRIIB on peripheral blood mononuclear cells (Figure 5B, left). Of the three potential founders identified (M27-1, F6-5, and F6-4; Figure 5B, left), only one (F6-5) showed germline transmission of the transgene. Transgenic descendants of F6-5 showed uniform expression of human FcγRIIB (Figure 5B) and were used for all further experiments.

We generated $_{cd11c}$FcγRIIB$^{hu}$ BMDCs and confirmed that they upregulated expression of MHC class II, CD86, CD40, and CD80 after LPS-triggered activation/ maturation (Figure 6A) and that CRP alone had no effect on expression of these markers (Figure 6A). Expression of human FcγRIIB partly reconstituted the CRP prohibitory effect on BMDC maturation, i.e., upon LPS-stimulation, CRP prohibited the expression of MHC class II and CD40 (Figure 6A). CRP inhibited IL-10 production by LPS-stimulated $_{cd11c}$FcγRIIB$^{hu}$ BMDCs (459.4 ± 3.1 ng/ml without CRP and no detectable amounts with CRP), but not IL-12p70 production (485.9 ± 94.8 ng/ml and 689.9 ± 235.9 ng/ml without or with CRP, respectively). Although the effect was not significant (ns), when $MOG_{35–55}$ peptide-loaded $_{cd11c}$FcγRIIB$^{hu}$ BMDCs were used as APCs, their ability to stimulate the proliferation of 2D2 T cells was reduced by CRP (Figure 6B). These data generally support the premise that CRP's influence on DCs requires their expression of FcγRIIB, since some of the effects of CRP on FcγRIIB$^{-/-}$ BMDCs are recovered by

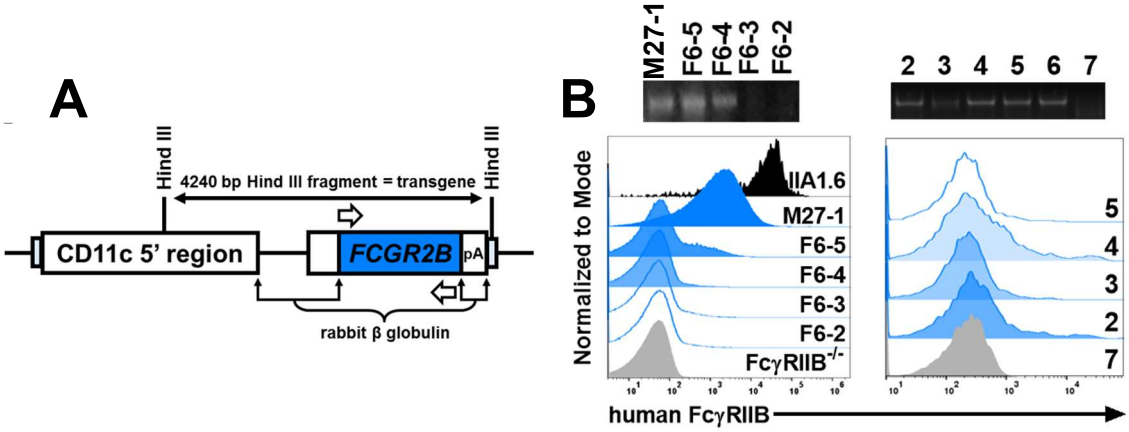


**Figure 5. Generation of $_{cd11c}$FcγRIIB$^{hu}$ mice.** (A) The targeting vector (fully described in 10) encodes the mouse CD11c minimal promoter driving the human FCGR2B open reading frame. (B) Agarose gel electrophoresis of PCR amplified gDNA from a male (M27-1) and 4 female (F6-2,-3,-4, and -5) potential founders; three of the mice carry the *FCGR2B* transgene as indicated by presence of a 658 base pair amplicon (black arrow) generated using human *FCGR2B*-specific primers (white arrows in panel A). Expression of human FcγRIIB on mouse peripheral blood cells, as detected by flow cytometry using an anti-human FcγRIIB antibody (clone AT 10). Murine B cell lymphoma IIA1.6 cells transfected with a plasmid containing cDNA encoding human FcγRIIB (fully described in reference 11) and peripheral blood cells from an FcγRIIB$^{-/-}$ mouse served as controls. The inset shows a representative flow cytometry histogram from mouse M27-1 (blue) and an FcγRIIB$^{-/-}$ mouse (gray).

expression of human FcγRIIB. Interestingly, although reconstitution of FcγRIIB$^{-/-}$ BMDCs with human FcγRIIB restored their ability to promote $OVA_{323–339}$-driven OT-II T cell proliferation, CRP (50 μg/ml) did not have a significant effect (data not shown).

*Human FcγRIIB Supports Human CRP's Protective Actions in EAE*

We had previously shown that CRPtg undergoing EAE have delayed onset and reduced severity of disease compared to WT and that this beneficial effect of CRP is FcγRIIB-dependent (5, 6, 20, 25), and herein we provide new evidence that this FcγRIIB-dependency extends to BMDCs *in vitro*. Moreover, although not all the observed effects of human CRP on BMDCs were supported by human FcγRIIB, CD11c-specific expression of human FcγRIIB was sufficient to fully reconstitute human CRP's beneficial actions in EAE (Figure 7; Table 1). Given that human CRP can utilize human FcγRIIB

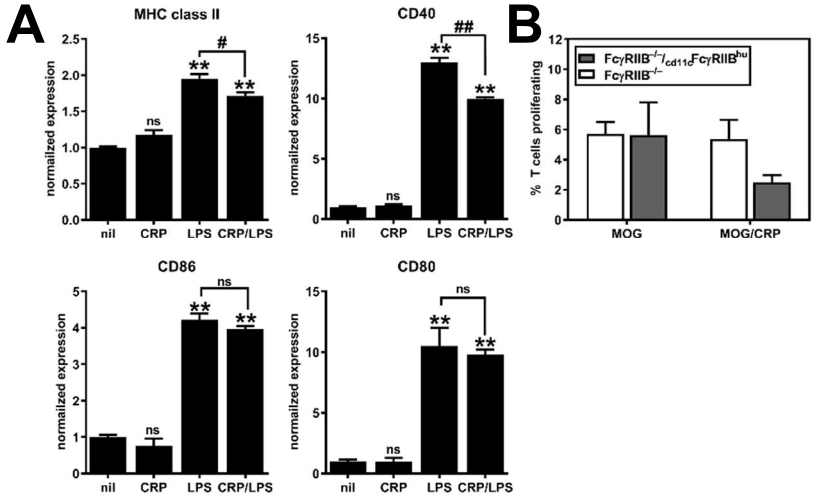


**Figure 6. CD11c-specific expression of human FcγRIIB reconstitutes CRP-mediated suppression of expression of MHC class II and CD40 on LPS-matured BMDCs (A) and CRP-mediated suppression of BMDC-mediated/MOG-driven 2D2 T cell proliferation (B).** Flow cytometry of CD11c$^{+}$ $_{cd11c}$FcγRIIB$^{hu}$ BMDCs and BMDC:2D2 T cell co-cultures. (A) Surface expression of MHC class II, CD86, CD40, and CD80 left untreated (nil),or treated with CRP (50 μg/ml), LPS (1 μg/ml) or CRP and LPS. Each marker expression is normalized to nil and the symbols directly above each bar indicate $p < 0.005$ (**) compared to nil. The symbols above each bracket indicate $p < 0.05$ (#), or $p < 0.005$ (##) comparing LPS vs LPS/CRP groups. (B) 2D2 T cell proliferation with $MOG_{35-55}$ peptide-loaded BMDCs without or with CRP (50 μg/ml). One-way ANOVA with Tukey's multiple comparisons test from n = 3 – 6.

expressed by CD11c$^{+}$ cells in transgenic mice, it is possible that the same or a similar CRP → FcγRIIB pathway operates in humans to regulate tolerance and prevent autoimmunity.

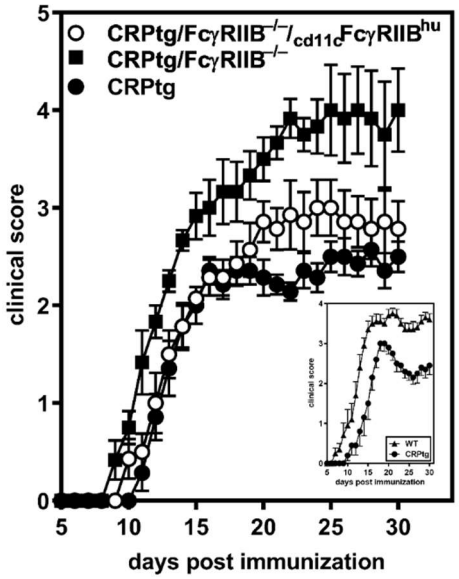


**Figure 7. CD11c-specific expression of human FcγRIIB restores resistance to EAE in CRPtg/FcγRIIB$^{-/-}$ mice.** Immunization of mice with $MOG_{35-55}$ and the ensuing EAE symptoms were monitored for 30 d in CRPtg (●), CRPtg/FcγRIIB$^{-/-}$ (□), and CRPtg/FcγRIIB$^{-/-}$/$_{cd11c}$FcγRIIB$^{hu}$ (○). Asterisk (*) or not significantly different (ns) is compared to CRPtg: see Table I for details. Inset: the course of EAE in CRPtg compared to WT from a separate experiment; n = 6 – 10 mice.

**Table 1. EAE outcomes in CRPtg lacking mouse FcγRIIB and/or expressing human FcγRIIB**

| Genotype (n) | day of onset[a] mean ± SEM | CDI[b] mean ± SEM | maximum score[c] mean ± SEM |
|---|---|---|---|
| CRPtg (7) | 14.0 ± 0.43 | 41.71 ± 0.94 | 2.86 ± 0.09 |
| CRPtg/FcγRIIB$^{-/-}$ (6) | 11.67 ± 0.33[d] | 67.17 ± 5.15[d] | 4.17 ± 0.40[d] |
| CRPtg/FcγRIIB$^{-/-}$/$_{cd11c}$FcγRIIB$^{hu}$ (7) | 13.71 ± 0.52[ns] | 48.43 ± 3.79[ns] | 3.43 ± 0.28[ns] |
| ANOVA | $p = 0.004$ | $p = 0.0005$ | $p = 0.0135$ |

[a] the day the clinical score attained a value ≥ 2 and remained ≥ 2 for at least 2 days.
[b] cumulative disease index: the sum of clinical scores from day 0-31.
[c] the maximum clinical score attained by each mouse. Mice that succumbed to EAE were assigned a score of 6.
[d] Tukey's multiple comparisons test, $p < 0.05$.
[ns] Tukey's multiple comparisons test, not significant ($p > 0.05$).

## Discussion

Previously, we showed that human CRP protects CRPtg mice from EAE triggered either directly by immunization with MOG or indirectly by the transfer of MOG-specific T cells (5, 20, 25) and that this protection was FcγRIIB-dependent manner (6). Although human CRP can have direct effects on T cells (25), the initial evidence of CRP inhibiting EAE suggested that CRP most likely conferred protection by acting on an intermediary APC. This study provides strong evidence to this effect, i.e., *in vivo* human CRP protects mice from EAE by acting on $CD11c^+$ FcγRIIB-expressing DCs. We propose that the beneficial effect of transgenically expressed human CRP in EAE, and perhaps other T cell-mediated diseases like lupus and collagen-induced arthritis (26–37), is achieved *via* its capacity to inhibit DC development and function, thereby diminishing the stimulation of pathogenic T cells. Our *in vitro* data reveal several separate, but likely additive, mechanisms by which CRP impacts the T cell stimulating actions of DCs. First, CRP dose-dependently decreased the proportion of BM progenitors that developed into BMDCs, suggesting that CRP influences the fate of hematopoietic stem cells. Native pentameric CRP is likely required for this effect as heat denatured CRP did not have any effect (data not shown). Furthermore, CRP did not significantly affect early or late apoptosis or necrosis during the course of BM culture, demonstrating that CRP binding to phosphatidylserine on dying cells does not play a significant role and that CRP's influence is likely not due to selective killing of certain BM progenitors. Indeed, in separate studies we have also observed that CRP dose-dependently promotes the development of myeloid-derived suppressor cells (MDSC) at the expense of DCs (Figure S1 in Supplementary Material), and that the spleens of healthy CRP knockout mice (27) have an increased number of plasmacytoid ($CD11c^+$ $CD11b^{+/-}$ Siglec $H^+$) and

conventional ($CD11c^+$ $CD11b^+$ Siglec $H^-$) DCs compared to WT and CRPtg (Appendix C, Supplemental Figure 2). The mechanism by which CRP alters myeloid progenitor cell developmental fate is still under investigation, but the fact that CRP shifts the myeloid balance away from DCs (which can promote T cell proliferation) and toward MDSCs (which can suppress it) directly implicates CRP in the regulation of the balance between adaptive immunity and tolerance. Second, CRP dose-dependently prohibits the LPS-triggered (TLR4-triggered) activation/maturation of BMDCs as evidenced by its ability to limit expression of MHC class II and co- stimulatory markers. Notably, CRP had no effect in the absence of a maturation signal (i.e., immature BMDCs) or in the presence of the TLR9 agonist CpG oligodeoxynucleotides (data not shown). This implies that *in vivo* CRP attenuates the responses of mature DCs in the periphery (i.e., those not participating in central tolerance) and does not impact immature DCs. Third, CRP impairs the production of IL-10 and IL-12p70 by BMDCs, two pleiotropic cytokines that can suppress (29) or promote (30) T cell functions, respectively. Fourth, CRP inhibited the ability of peptide-loaded mature BMDCs to stimulate antigen-driven T cell proliferation. Unexplored was whether CRP impairs the ability of BMDCs to uptake, process, and present antigen, but others have shown that CRP can also impact these processes (31–33). We previously showed that in the absence of FcγRIIB, human CRP cannot protect mice against EAE (6). That observation led us here to test whether the CRP-responsive, FcγRIIB-expressing cell that might promote CRP's beneficial effects in EAE are DCs (7). In preliminary studies, we showed that CRP dose-dependently decreased the yield of both WT and $FcR\gamma^{-/-}$ $CD11c^+$ BMDCs, but not $Fc\gamma RIIB^{-/-}$ ones (Appendix C, Supplemental Figure 3). In alignment with those initial data, we showed herein that

FcγRIIB$^{-/-}$ BMDCs maintain their ability to mature in response to LPS and to subsequently stimulate 2D2 T cell proliferation when loaded with $MOG_{35–55}$, but are refractory to inhibition by CRP. Importantly, in the absence of FcγRIIB expression, CRP was unable to downregulate BMDC production of the T cell stimulating cytokine IL-12p70. These findings highlight the importance of FcγRIIB for the inhibitory action of CRP on the development, maturation, cytokine production, and antigen-specific T cell stimulatory function of BMDCs. Since, human CRP can bind both mouse and human FcγRs *in vitro* and *in vivo* (2, 21), we generated a mouse completely deficient in endogenous mouse FcγRIIB, but expressing human *FCGR2B* on CD11c$^{+}$ cells. Using bone marrow from these cd11cFcγRIIBhu mice we showed that human CRP utilized human FcγRIIB to evoke impairment of BMDC activation and T cell stimulating function, but not to regulate IL-12p70 production. Nevertheless, CRP protection from EAE was fully reconstituted in CRPtg/$_{cd11c}$FcγRIIB$^{hu}$ mice. We recognize that mouse CD11c, and, therefore, human FcγRIIB in the $_{cd11c}$FcγRIIB$^{hu}$ mice, might be expressed at low levels on cell types other than DCs and that other DC subtypes may not express CD11c at all [e.g., plasmacytoid DCs and DCs with tolerogenic phenotypes (34)]. Nevertheless, this study is the first to show that human CRP interaction with human FcγRIIB expressed *in vivo* on CD11c$^{+}$ cells can modulate EAE. We suspect that CRP regulates the generation and actions of DCs in the periphery (i.e., those not directly involved in central tolerance), thereby limiting the activation of auto-reactive T cells especially in the setting of tolerance breakdown. Withal, CRP promotes the number and generation of myeloid-derived suppressor cells (MDSCs) [Appendix C, Supplemental Figure 1 (35)], a cell type known to potently suppress T cell proliferation (36). Simply by

modulating the myeloid lineage development away from DCs and toward MDSCs, CRP could thus profoundly impact T cell immunity and the maintenance of peripheral tolerance. This role is unlikely to be restricted to EAE/MS and should also be manifest in the setting of immunosenescence and aging, for example [reviewed in Ref. (37)]. Indeed, some of the prominent features of immunosenescence are inflammation, decreased T cell numbers, and decreased naïve and memory T cell responsiveness (37, 38, 39), and in the aged, inflammaging can contribute to dysregulated DC responses and a consequent breakdown of tolerance that can predispose them to autoimmunity (40, 41). We propose that in this context, modest elevation of CRP due to biological aging (12) might act as a tonic suppressor of DC activation and thus limit auto- reactivity.

## Ethics Statement

This study was carried out in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals; Eighth Edition* (NIH Academies Press, 2011) and the Institutional Animal Care and Use Committees at UAB.

## Author Contributions

AS, RJ, TW, and NJ designed the experiments and RJ, TW, and NJ performed them. JW and AG aided $_{cd11c}$FcγRIIB$^{hu}$ mouse generation. RJ and AS wrote the manuscript.


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## Supplementary Material

The Supplementary Material for this article can be found in APPENDIX C, page 149.

## Conflict of Interest Statement

The authors declare that the research was con-ducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

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# C-REACTIVE PROTEIN PROMOTES THE EXPANSION OF MYELOID DERIVED CELLS WITH SUPPRESSOR FUNCTIONS

by

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Format adapted and errata corrected for dissertation

## Abstract

Previously we established that human C-reactive protein (CRP) exacerbates mouse acute kidney injury and that the effect was associated with heightened renal accumulation of myeloid derived cells with suppressor functions (MDSC). Herein we provide direct evidence that CRP modulates the development and suppressive actions of MDSCs in vitro. We demonstrate that CRP dose-dependently increases the generation of MDSC from wild type mouse bone marrow progenitors and enhances MDSC production of intracellular reactive oxygen species (iROS). When added to co-cultures, CRP significantly enhanced the ability of MDSCs to suppress CD3/CD28-stimulated T cell proliferation. Experiments using MDSCs from FcγRIIB deficient mice (FcγRIIB$^{-/-}$) showed that CRP's ability to expand MDSCs and trigger their increased production of iROS was FcγRIIB-independent, whereas its ability to enhance the MDSC T cell suppressive action was FcγRIIB-dependent. Importantly, CRP also enabled freshly isolated primary human neutrophils to suppress proliferation of autologous T cells. These findings suggest that CRP might be an endogenous regulator of MDSC numbers and actions in vivo.


## Introduction

Human C-reactive protein (CRP) is the prototypical acute phase reactant; CRP serum levels can rapidly increase from typically ≤ 3 μg/ml at baseline to upwards of 500 μg/ml in response to proinflammatory cytokines produced during inflammation (e.g., IL-6, IL-1β, and TNFα) (1, 2). The human CRP molecule is a planar, pentameric pattern-recognition receptor with a high affinity for phosphocholine (3) that can function as an

opsonin (4, 5), activate the classical pathway of complement (6), and bind to various Fc receptors (FcR) thereby triggering effector responses like phagocytosis and cytokine secretion (7, 8). Its wide-ranging blood levels and sensitivity to inflammation make human CRP a useful clinical biomarker of diseases such as cardiovascular, autoimmune, and Alzheimer's disease (9, 10). For example, CRP levels are often monitored in patients with acute kidney injury (AKI) wherein they correlate with increased AKI risk, severity, and clinical outcomes (11–13). Importantly, our group recently established that expression of human CRP (by CRP transgenic mice; CRPtg) exacerbated renal ischemia reperfusion injury, an experimental model of AKI (14). Notably, the detrimental action of CRP was associated with an increased renal accumulation of myeloid cells with a suppressor phenotype (hereafter, MDSC).

Moreover, we showed that antibody-mediated depletion of MDSCs alleviated renal injury in CRPtg and that targeted lowering of human CRP, which led to diminished MDSC renal accumulation, lessened the severity of AKI in CRPtg (15). Neither mouse nor human MDSCs have a unique marker and their exact origins remain equivocal; however, there is a growing consensus that MDSCs are a heterogeneous group of immature and highly proliferative cells that arise in various pathological states (16, 17). As their name implies, MDSCs potently suppress the proliferation of cells in their immediate vicinity; suppression of T cell proliferation being the gold standard by which this is assessed. The suppressive action of MDSCs is thought to be the consequence of their ability to deplete the essential amino acids arginine (achieved via MDSC expression of arginase) and tryptophan (achieved via MDSC expression of indolamine-2,3-dioxygenase), and by their robust production of reactive nitrogen and oxygen species

(RNS and ROS, respectively) (18). Although MDSCs were initially described as key mediators of immune suppression during tumorigenesis (19), it is increasingly evident that MDSCs also participate during trauma (20, 21) and sepsis (22, 23).

To understand how human CRP might impact the biology of MDSCs, and thereby better understand the sequence of events that leads to worsening of AKI in CRPtg mice, herein we used mouse bone marrow cultures to directly interrogate the impact of human CRP on mouse MDSC development and suppressive actions. Since any observed effect of human CRP on mouse MDSCs might be an aberration of the xenogeneic (i.e., human protein/mouse cell) system, we also performed studies using freshly isolated primary human myeloid cells. Our results show that in the presence of human CRP, mouse bone marrow myeloid progenitor cell commitment is biased toward MDSCs and away from dendritic cells (DC). Furthermore, human CRP triggers the generation of iROS by mouse MDSCs and enhances their ability to suppress the proliferation of CD3/CD28 stimulated mouse CD4+ T cells. Interestingly, human CRP triggered enhancement of the immune suppressive action of mouse MDSCs is FcγRIIB-dependent, but its ability to stimulate iROS is not. Human CRP also augmented the production of iROS by freshly isolated human peripheral blood neutrophils and enabled them to exert a suppressive effect on the proliferation of autologous human T cells. Our findings demonstrate that CRP might be an endogenous regulator of MDSCs and suggest that monitoring and/or targeting CRP might be a useful clinical strategy for a growing list of pathologies in which MDSCs are known to participate.

## Materials and Methods

### *Mice*

All animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the *Guide for the Care and Use of Laboratory Animals; Eighth Edition* (NIH Academies Press, 2011). All mice used were from the C57BL/6 background, housed in the same vivarium at constant humidity (60 ± 5%) and temperature (24 ± 1◦C) with a 12 h light cycle (6 a.m.− 6 p.m.), and maintained *ad libitum* on sterile water and regular chow (Harlan Teklad). Mice were at least 8 weeks old when bone marrow, spleens, and lymph nodes were harvested and both sexes were used. Where indicated, bone marrow (BM) was harvested from FcγRIIB deficient mice (FcγRIIB$^{-/-}$; B6.129S4-Fcgr2b$^{tm1TtK}$ N12, Taconic Farms model 580) that lack functional expression of the gene encoding the α-chain of mouse FcγRIIB (24).

### *Generation of Mouse Myeloid Derived Suppressor Cells*

Mouse bone marrow myeloid derived suppressor cells (BM-MDSC) were generated as described by Höchst et al. (25). Briefly, BM was flushed from mouse femurs and tibias using a Hank's Balanced Salt Solution (HBSS; Gibco) filled 1 ml syringe fitted with a 25G × 5/8″ needle. The recovered bone marrow was strained through a nylon filter (70 μm) and erythrocytes were lysed (Hybri-Max Red Blood Cell Lysing Buffer; Sigma R7757). Filtered BM cells were suspended in Minimum Essential Medium Eagle—Alpha Modification (αMEM; Lonza 12-169F) supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS, Gibco 10082147), 2 mM GlutaMAX (Gibco 35050061), 100U

per ml/100 μg per ml penicillin/streptomycin (Gibco 15140122), 1 mM sodium pyruvate (Gibco 11360070), 55 μM β-mercaptoethanol (Gibco 21985023), and 40 ng/ml mouse granulocyte/macrophage-colony stimulating factor (GM-CSF; Shenandoah Biotechnology 200-15) and then seeded into 12-well tissue culture-treated plates ($1\times10^6$ cells/well) and grown (37°C, 5% CO2) for 96 h (i.e., d4) unless otherwise noted. The culture medium was changed at 72 h (d3). Highly purified (~95%) human CRP from pleural/ascites fluids (US Biological Sciences C7907-26A) was filtered (0.2 μm) and diluted in Tris-buffered saline pH 7.4 without preservatives, and added at the start of culture (d0) and with the media change at 72 h. Prior to use, samples of human CRP were subjected to polyacrylamide gel electrophoresis to confirm its integrity (data not shown). On d4, cells were harvested with a cell scraper for cytometry analysis or used in downstream experiments as otherwise described.

To negatively enrich MDSCs, BM-MDSCs (d4 cultures) were subjected to separation using the EasySep mouse CD11c positive selection kit II (StemCell Technologies 18780) according to the manufacturer's instructions. This approach effectively removed the contaminating $CD11c^+$ fraction (i.e., DCs), yielding a highly purified (94 ± 1.5%) $CD11c^-$ MDSC fraction (Appendix D, Supplemental Figure 1). For experiments utilizing $Fc\gamma RIIB^{-/-}$ BM-MDSCs, wild type BM-MDSCs were grown concomitantly and used simultaneously in suppression assays or ROS assays.

*Cell Cycling Analysis by Bromodeoxyuridine Incorporation*

To assess cell cycling d4 BM-MDSC were exposed to human CRP for 24 h, with 20 μM bromodeoxyuridine (BrdU; Sigma B5002) added 3 h prior to harvesting cells with a cell scraper. BM-MDSCs were then fixed using pre-chilled 70% ethanol added

dropwise while vortexing. After incubation for 20 min. the DNA was linearized by adding 2 N HCl while vortexing. After incubation for 20 min. the cells were permeabilized with 0.1 M $Na_2B_4O_7$ for 2 min. Next, non-specific binding was blocked by 15 min incubation (4°C) with anti-CD16/CD32 monoclonal antibody (mAb clone 93; eBioscience 14-0161-82), and finally BrdU incorporation was probed using of APC conjugated anti-BrdU antibody (clone Bu20a, BioLegend 339808) for 30 min at 4°C (4 µl/tube). Ten minutes prior to cell cytometry total DNA was stained with 1 µg of 7-aminoactinomycin D (7-AAD, Invitrogen A1310). Cell cytometry was performed on a BD LSRII cytometer using BD FACSDiva version 6.1.3 software, a standard gating strategy was used to identify cells in the G0/G1, S, and G2/M phases of cell division [adapted from (26)], and the acquired data was analyzed using FlowJo version 10.3. Briefly, single cells were gated on using an SSC-A × SSC-H dot plot and apoptotic cells (7-AAD$^{lo}$) were excluded. Untreated live cells were used to gate on cells in the G0/G1 (7-AAD$^{int}$BrdU$^{lo}$), S (7-AAD$^{lo-hi}$BrdU$^{+}$), and G2/M (7-AAD$^{hi}$BrdU$^{+}$) phases of cell division. Cells in S phase were further subdivided into three subpopulations corresponding to cells in early (7-AAD$^{lo}$BrdU$^{+}$), middle (7-AAD$^{int}$BrdU$^{+}$), and late S phase (7-AAD$^{hi}$BrdU$^{+}$).

*Mouse MDSC-Mediated Mouse T Cell Suppression Assays*

To isolate mouse CD4$^{+}$ T cells the spleen and lymph nodes (inguinal, axillary, brachial) from wild type mice were mechanically homogenized, erythrocytes were lysed, and the resultant homogenate filtered (70 µm). The single cell suspension was then subjected to negative selection using the EasySep mouse CD4$^{+}$ T cell isolation kit (StemCell Technologies 19852) according to the manufacturer's instructions. Negatively enriched CD4$^{+}$ T cells were then stained with 0.5 µM carboxyfluorescein succinimidyl

ester (CFSE; Invitrogen 65085084) in PBS for 20 min at room temperature, washed and re-suspended in RPMI 1640 media (Gibco 11875119) supplemented with 5% HI-FBS, 2 mM GlutaMAX, 100 U/ml penicillin, 100 μg/ml/streptomycin, 1X MEM non-essential amino acids (Gibco 219850232) and 55μM β-mercaptoethanol. Mouse CFSE+CD4+ T cells were then added to a tissue culture-treated 96-well plate ($2 \times 10^5$ cells/well) coated with 2 μg/ml of anti-CD3ε mAb (functional grade, clone 145-2C11; Invitrogen 16-0031-82) in the presence of 1 μg/ml soluble anti-CD28 mAb (functional grade, clone 37.51; Invitrogen 16-0281-81). After 72 h (d3), mouse $CD4^+$ T cells were harvested and their proliferation (CFSE dilution) was assessed by flow cytometry (Appendix C, Supplemental Figures 2A,B). Prior to performing T cell suppression assays each lot of anti-CD3ε mAb was titrated and used at concentrations that resulted in 3 – 5 discernable generations of $CFSE^+CD4^+$ T cells after 72 h of culture (Appendix D, Supplemental Figure 2B). When studying the effects of MDSCs on T cell proliferation MDSCs were from d4 cultures and they were added to the T cells to achieve effector:target (E:T) ratios ranging from 10E:1T to 1E:20T.When studying the effects of CRP on MDSC-mediated suppression of T cell proliferation CRP (1 – 100μg/ml) was added only at the beginning of co-culture.

Proliferation of mouse $CFSE^+CD4^+$ T cells was assessed and is reported following standard conventions detailed by Roederer (27). Thus, (i) when non-proliferated and proliferating generations were discrete and easily discerned (e.g., Figure 3D) we calculated the proliferation index, i.e., a ratio of the average number (across biological and technical replicates) of generations of proliferating T cells normalized to the maximum number of generations of proliferating T cells when they were cultured in

isolation, (ii) when non-proliferated cells were in excess (e.g., Figure 3D) we calculated the division index, i.e., the average number of cell divisions carried out by all the T cells in co-cultures, normalized to their maximum number of cell divisions when they were cultured in isolation, and (iii) in cases where proliferating generations of T cells were difficult to resolve (due to high intergeneration variance or high autofluorescence; e.g., Figure 5C) we calculated the fraction diluted, i.e., we averaged the fraction of T cells in the final culture that divided at least once and normalized this to maximum proliferation achieved by T cells cultured in isolation or to co-cultures without human CRP added, depending on the experiment. Proliferation and division indexes and the fraction diluted were calculated using CFSE dilution histograms normalized to mode (the most populous T cell generation) as defined by FlowJo version 10.3. In all cases, the experiments were conducted with technical triplicates to ensure the rigor of the co-culture system.

*Human Neutrophil-Mediated Human T Cell Suppression Assays*

Under the auspices of protocols approved by the Institutional Review Board of the University of Alabama at Birmingham, in accordance with the recommendations of the Belmont Report, and after subjects gave written informed consent, neutrophils were purified from the whole blood of healthy adult human donors using the EasySep Direct Human Neutrophil Isolation Kit (StemCell Technologies 19666) according to the manufacturer's instructions. Concurrently, autologous human $CD3^+$ T cells were isolated from PBMCs by immunomagnetic negative selection using EasySep Human T Cell Isolation Kit (StemCell Technologies 17951) according to the manufacturer's protocol. The isolated $CD3^+$ T cells were stained with 1.25 μM CFSE (Invitrogen C34554) in PBS for 8 min at 37°C. To stimulate their proliferation, $5 \times 10^4$ $CFSE^+CD3^+$ human T cells in

RPMI1640 (Corning 10-040-CM) supplemented with 10% heat inactivated human serum type AB (Atlanta Biologicals S40110) and 1X penicillin/streptomycin (Corning 30-002-CI) were added to a 96-well plate coated with 5 μg/ml anti-CD3ε mAb (BioLegend 300401) and 2 μg/ml soluble anti-CD28 mAb (BioLegend 302901). Autologous neutrophils were added to the T cells to achieve a 1:1 E:T ratio and the cells were incubated at 37°C in 5% $CO_2$ for 72, 96, or 120 h. Human $CD3^+$ T cell proliferation (CFSE dilution) was recorded on an Invitrogen Attune NxT flow cytometer and quantitated as described for mouse T cells.

*Flow Cytometry of Mouse Cells*

For mouse BM-MDSC phenotyping and mouse T cell suppression assays single cell suspensions were stained with eFluor780 viability dye (eBioscience 65-0865) for 30 min at room temperature, fixed with 0.5X Fixation Buffer (BioLegend 420801) for 10 min at room temperature, and non-specific binding was blocked with an anti-CD16/CD32 mAb (clone 93; eBioscience 14-0161-82) for 15 min at 4°C. Cells were then stained for 30 min at 4°C with specific fluorochrome-labeled antibodies (all from BioLegend). Mouse BM-MDSCs were stained using anti-mouse CD11b (clone M1/70), CD11c (clone N418), F4/80 (clone BM8), Ly6C (clone HK1.4), and Ly6G (clone 1A8). Mouse T cells were stained with anti-mouse CD4 (clone RM4-5). After staining the cells were washed and suspended in PBS and cytometry performed on a BD LSR-II cytometer equipped with BD FACSDiva version 6.1.3. The acquired data were analyzed with FlowJo version 10.3. Debris, doublets, and $eFlour780^+$ dead cells were gated out before any assay-specific gating (gating strategy shown in Appendix D, Supplemental Figure 2A).

*Assessment of Reactive Oxygen Species Production*

To measure extracellular ROS, mouse BM-MDSCs (d4) were harvested or primary human neutrophils were isolated and added ($5 \times 10^5$/well) to a white 96-well plate (Corning 3355) containing 200 μM luminol (Sigma A8511) and 1.6 U/ml of horseradish peroxidase (Sigma P2088). The cells were thereafter left untreated (HBSS control) or treated with human CRP or 100 nM phorbol 12-myristate 13-acetate (PMA) and the amount of oxidized luminol (luminescence units, LU) measured immediately and for up to 60min thereafter on a Bio-Tek Synergy 2 with Gen5 version 1.10. For each condition the background signal (the first LU reading) was subtracted from all subsequent readings, and for PMA- and CRP-treated cells the data were normalized to their genotype-matched PBS controls (relative LU, RLU). To measure iROS, $5 \times 10^5$ enriched mouse MDSCs (WT or FcγRIIB$^{-/-}$) or primary human neutrophils were added to the wells of a tissue culture-treated, clear-bottom, black-sided 96-well plate (Greiner Bio-One 65509099) loaded with freshly reconstituted 2.5 μM 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA, Invitrogen D399) at 37°C in 5% $CO_2$ for 30 min. $H_2$DCFDA is cell-permeant and cleaved intracellularly, preventing its exit, and specifically fluoresces upon oxidation by iROS. Thereafter the cells were left untreated (PBS control) or treated with human CRP or 100 nM PMA and fluorescence intensity (FI; excitation 485 nm/emission 535 nm) was immediately measured and for 60 – 180 min thereafter on a Tecan Infinite M200 Pro using i-control version 1.7.1.12. For each condition the background signal (the first FI reading) was subtracted from all subsequent readings, and for PMA- and CRP-treated cells the data were normalized to their genotype-matched HBSS/PBS controls (relative FI, RFI).

*Statistical Analysis*

Raw data from biological replicates (experiments using BM cultures from different mice) and technical replicates (experiments repeated using a single BM cultures) were pooled as appropriate and the means with associated SEMs or SDs is presented. Group comparisons were done using one-way or two-way analysis of variance (ANOVA) followed by post-hoc Dunnett's or Sidak's multiple comparisons tests, or using 1-tailed Student's t-tests (un-paired or paired as appropriate). Differences were considered significant when the test $p \leq 0.05$. To estimate CRP's potency we employed non-linear regression to estimate the concentration of CRP required to enhance MDSC mediated suppression of T cell proliferation by 50% ($IC_{50}$). All statistical and regression analyses were done using GraphPad Prism version 7.00.

## Results

*Human CRP Promotes the Generation of Mouse MDSCs*

Mouse bone marrow (BM) was grown under conditions previously shown to expand myeloid derived cells into cells with suppressor functions [i.e., BM-MDSCs; protocol adapted from Höchst et al. (25)]. After 4 days in culture the majority of cells recovered either retained an immature myelocyte mononuclear appearance or had ring-shaped nuclei, while fewer had a polymorphonuclear appearance typical of mature granulocytes (Figure 1A); this heterogeneity is consistent with the reported range of nuclear morphologies characteristic of MDSCs found in vivo in both mice and humans (28). To determine the impact of CRP on the growth of mouse bone marrow progenitors, human CRP was added to cultures and their cell cycling was assessed by flow cytometry

after BrdU/7-AAD incorporation. We found that the addition of human CRP increased the frequency of cells entering early S-phase (Figure 1B); this effect was dose-dependent and achieved statistical significance at a dose of 100 μg/ml of CRP (Figure 1C). Additional experiments showed that the CRP dependent increase in the number of cells entering S-phase was not an artifact due to selective culling of cells exposed to CRP, as CRP treatment had no statistically significant effect on (i) the total number of cells recovered on day 4 of culture, (ii) the proportion of apoptotic and necrotic cells on day 4 of culture, and (iii) the overall viability of cells recovered on day 4 of culture (assessed by hemocytometer counts of trypan blue negative cells, flow cytometry frequencies of Annexin $V^{+/-}$7-$AAD^{+}$ cells, and flow cytometry frequencies of viability dye $eFlour780^{lo}$ cells, respectively) (data not shown).

By flow cytometry the majority of cells in mouse BM-MDSC cultures (66.3 ± 3.2% of cells recovered from n = 12 cultures) displayed a $CD11b^{+}CD11c^{-}F4/80^{-}Ly6G^{+}$ $Ly6C^{+}$ MDSC surface phenotype (Figures 1D,E); 84.6 ± 5.0% of these were of the $Ly6G^{+}Ly6C^{+}$ polymorphonuclear MDSC subtype (PMN-MDSC) and 12.0 ± 6.7% were the $Ly6G^{-}Ly6C^{+}$ monocytic MDSC subtype (M-MDSC) (Figure 1F). The remaining cells in d4 cultures were $CD11b^{+}CD11c^{+}$ DCs (24.0 ± 10.5% of all cells in culture) or $CD11b^{+}$ $CD11c^{-}F4/80^{+}$ macrophages (1.7 ± 1.5% of all cells in culture) (Figures 1D–F). As there is no accepted marker for MDSCs, we determined that d4 cultures contained a preponderance of MDSCs as verified by direct hemocytometer counts of fractions captured vs. not captured by CD11c positive immunomagnetic selection (fractionations of n = 7 separate cultures). By this approach it was estimated that 76.7 ± 7.1% of all cells in the d4 cultures were $CD11c^{-}$ MDSCs (Appendix D, Supplemental Figure 1), (66.3 ± 3.2%).

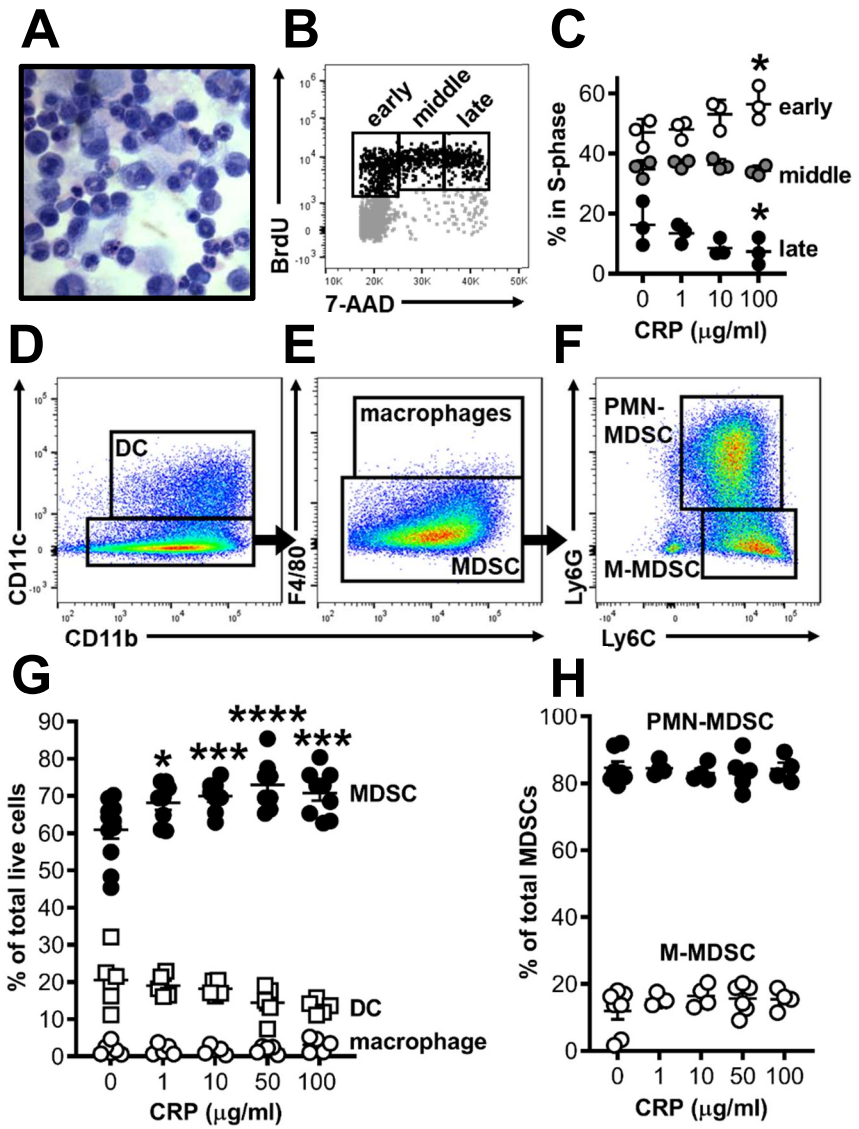


**Figure 1. Human CRP Promotes Expansion of Mouse MDSCs.** Mouse bone marrow cells were cultured under conditions tailored to generate MDSCs. (A) After 4 days of culture (d4) the majority of cells retained immature nuclear morphologies (light micrograph of Wright-Giemsa and hematoxylin stained cytospin; 100 X). (B) On d4 of mouse BM-MDSC culture human CRP was added and 24 h later, BrdU was added to incorporate for 3 h to identify cells in early, middle, and late S-phase of cell division (representative BrdU gating on untreated BM-MDSCs). (C) Human CRP dose-dependently increased entry of cells into early S-phase. The asterisks indicate $p < 0.05$ compared to 0 CRP (two-way ANOVA with Dunnett's multiple comparisons) for the representative mean ± SD of 2 experiments conducted in triplicates. (D – F) Flow cytometry gating strategy for identification of $CD11c^+CD11b^+$ DCs, $CD11b^+CD11c^-F4/80^+$ macrophages, and $CD11c^-CD11b^+F4/80^-Ly6G^+Ly6C^+$ MDSCs; MDSCs were further subtyped as PMN-MDSC ($CD11b^+CD11c^-F4/80^-Ly6G^+Ly6C^+$) or M-MDSC ($CD11b^+CD11c^-F4/80^-Ly6G^-Ly6C^+$). (G, H) Human CRP dose-dependently promoted MDSC expansion (G) but did not affect the relative distribution of MDSC subtypes (H). In panels G and H, the means ± SEM of n = 6 – 11 cultures are shown. The asterisks indicate *$p < 0.01$, ***$p < 0.005$, and ****$p < 0.0001$, compared to 0 CRP within each cell population (two-way ANOVA with Dunnett's multiple comparisons).

Importantly, after immunomagnetic removal of contaminating $CD11c^+$ cells the remaining cells were ~94% pure MDSCs as determined by flow cytometry (Appendix D, Supplemental Figures 1E,F). When added to the BM cultures, human CRP significantly and dose-dependently increased the proportion of MDSCs generated (Figure 1G). Notably the observed increase in MDSCs was at the expense of DCs, whose numbers were decreased by addition of CRP [as we have described elsewhere (29)]. Accordingly, the observed CRP dependent increase in the number of BM cells entering S-phase (Figures 1B,C) can be explained by CRP's selective enhancement of MDSC proliferation. However, although human CRP selectively promoted MDSC generation (Figure 1G), CRP had no effect on the relative proportion of PMN- vs. M-MDSCs (~85 and ~15%, respectively; Figures 1F,H). These data show that under conditions known to expand MDSCs from BM precursors, CRP selectively potentiates the expansion of cells with an MDSC surface phenotype.

*Human CRP Augments Mouse MDSC Production of Intracellular Reactive Oxygen Species*

MDSCs are prolific producers of ROS and this supports their capacity to strongly suppress the proliferation of T cells (18). Using a luminol based assay we confirmed that mouse BMMDSCs generated ROS robustly and in a biphasic pattern when stimulated with PMA (Figure 2A); this likely reflects an initial respiratory burst followed by sustained ROS production. In contrast, mouse BM-MDSCs stimulated with human CRP showed only a monophasic increase in ROS without evidence of a respiratory burst after (Figure 2A), and human CRP did not augment the respiratory burst triggered by PMA (data not shown). We also used the cell-permeant dye H2DCFDA to specifically measure

the production of intracellular ROS (iROS). Using this approach, we found that human CRP (50 and 100 μg/ml) significantly increased iROS production by enriched mouse MDSCs (Figure 2B). These data show that the mouse BM-MDSCs we generated are capable of robustly producing ROS, and that human CRP at concentrations seen during inflammation (2) specifically increases their iROS.

*Human CRP Augments Mouse MDSC Mediated Immune Suppression*

To establish that the BM-MDSCs we generated are bona fide suppressor cells and to test if CRP influences their suppressive activity, we used mouse BM-MDSCs as effector cells (E) in co-culture assays with CD3/CD28 stimulated target (T) mouse $CD4^+$ T cells. At E:T ratios of 1:1, 5:1, and 10:1, unfractionated BM-MDSCs significantly

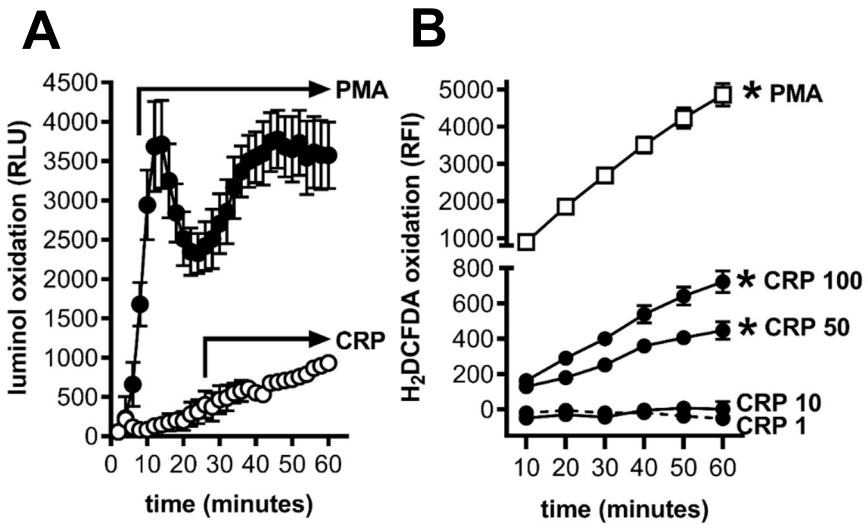


**Figure 2. Human CRP triggers intracellular ROS generation by mouse MDSCs.** (A) In luminol assays, mouse BM-MDSCs stimulated with 100 nM PMA exhibited an initial respiratory burst and subsequently sustained ROS production. In contrast, BM-MDSCs stimulated with CRP (100 μg/ml) exhibited only a monophasic increase in ROS. In each case the horizontal arrow begins at the first time point when luminol oxidation, relative luminescence units (RLU), was significantly elevated compared to untreated cells. (B) Detection of iROS production by enriched mouse MDSCs with the cell-permeant redox-sensing dye $H_2$DCFDA. Enriched mouse MDSCs exhibited a significant increase in iROS-dependent fluorescence intensity (FI) when stimulated with 100 nM PMA and high concentrations of human CRP. The arrows in A and the asterisks in B indicate $p < 0.05$ compared to untreated cells (two-way ANOVA with Sidak's multiple comparisons) of the representative mean ± SEM of 3 experiments conducted in 3 – 5 replicates.

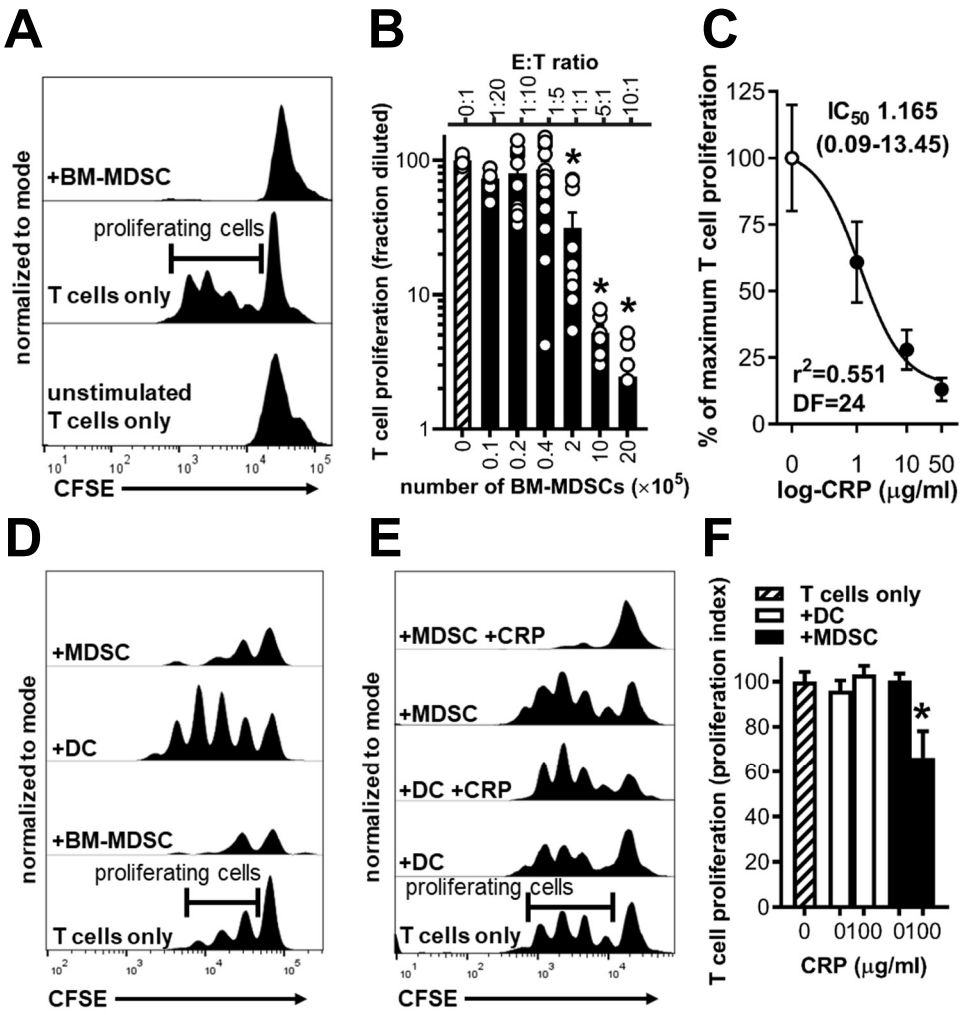


**Figure 3. Human CRP enhances the suppressive capacity of mouse MDSCs.** (A) Representative CFSE dilution histograms for $CD4^+$ T cells cultured in isolation (bottom trace), in the presence of anti-CD3 and anti-CD28 mAbs (middle trace), or in the presence of anti-CD3 and anti-CD28 mAbs plus BM-MDSCs (top trace; 10:1 E:T). (B) Results obtained from experiments shown as in panel A, but with increasing numbers of BM-MDSCs (*i.e.* increasing E:T ratios). The asterisks indicate $p < 0.0001$ compared to T cells alone (gray bar; one-way ANOVA with Dunnett's multiple comparisons) for the mean + SEM of n = 2 – 5 co-cultures conducted in triplicate. (C) In co-cultures employing 5:1 E:T, 100 μg/ml human CRP dose-dependently increased the ability of mouse BM-MDSCs to suppress T cell proliferation. The asterisks indicate $p < 0.005$ compared to co-cultures with 0 CRP (one-way ANOVA with Dunnett's multiple comparisons) for the mean of 2 co-cultures conducted in triplicate. (D) Representative CFSE dilution histograms comparing the mouse $CD4^+$ T cell suppressive capacities of mouse BM-MDSCs to selected mouse DCs vs enriched mouse MDSCs in co-cultures using a 1:20 E:T ratio. Only enriched mouse MDSCs (top trace) were suppressive. (E) Representative CFSE dilution histograms for mouse $CD4^+$ T cells in co-culture with selected mouse DCs vs enriched mouse MDSCs as in panel D, but with and without addition of human CRP (100 μg/ml). Only co-cultures using enriched mouse MDSCs plus human CRP (top trace) suppressed mouse $CD4^+$ T cell proliferation. (F) Proliferation indices for the representative experiment shown in panel E. The asterisk indicates $p < 0.05$ compared to co-cultures with 0 CRP (two-way ANOVA with Sidak's multiple comparisons) for the representative mean + SD of 3 co-cultures conducted in triplicate.

suppressed the proliferation of T cells (Figures 3A,B). Importantly, at a 5:1 E:T ratio human CRP dose-dependently augmented mouse BM-MDSC mediated suppression of mouse $CD4^+$ T cell proliferation with an $IC_{50}$ of 1.165 μg/ml (Figure 3C). In the absence of BM-MDSCs human CRP (≤ 100 μg/ml) had no discernable effect on the proliferation of mouse $CD4^+$ T cells (Appendix D, Supplemental Figure 2C).

To verify that the observed suppression of $CD4^+$ T cell proliferation was attributable to the action of MDSCs per se and not to other potentially suppressive cells present in the mouse BM-MDSC cultures, we compared the suppressive capacity of DCs ($CD11c^+$ cells captured by immunomagnetic selection) vs. enriched MDSCs ($CD11c^-$ cells left behind after immunomagnetic selection that are ~94% pure MDSCs as shown in Appendix D, Supplemental Figures 1E,F) from the same BM cultures. We found that after their enrichment in this way, mouse MDSCs were capable of fully suppressing mouse $CD4^+$ T cell proliferation even at an E:T ratio of 1:20 (Figure 3D). In stark contrast, at a 1:20 E:T ratio the DCs promoted T cell proliferation rather than suppressed it (Figure 3D). Importantly, human CRP (100 μg/ml) significantly enhanced the suppressive actions of enriched mouse MDSCs but had no significant effect on the actions of mouse DCs (Figures 3E,F). These data confirm that the mouse myeloid derived cells we generated are bona fide MDSCs and that human CRP selectively enhances their suppressive capacity.

*In the Absence of FcγRIIB Human CRP Does Not Augment Mouse MDSC Mediated Immune Suppression*

Many of the reported effects of human CRP on myeloid cells in vitro and in vivo have been attributed to CRP utilization of various FcRs, and there is much evidence for CRP utilizing the inhibitory Fc gamma receptor IIB (FcγRIIB, CD32B) (7, 8, 30). Since FcγRIIB can operate in trans to inhibit the actions of activating receptors like FcγRI (CD64) and FcγRIII (CD16) (31, 32), we used bone marrow from FcγRIIB$^{-/-}$ mice (24) to determine whether CRP-mediated augmentation of mouse MDSC expansion, iROS production, and CD4$^{+}$ T cell suppressive function might require FcγRs. First we verified that wild type mouse MDSCs express FcγRI, IIB, and III (flow cytometry data not shown), confirmed that cultures of FcγRIIB$^{-/-}$ BM yielded similar cell numbers with comparable viability compared to wild type BM cultures (Table 1) and that the relative proportions of MDSCs, DCs, and macrophages generated in FcγRIIB$^{-/-}$ BM cultures was similar to that in wild type BM cultures (Figure 4A), and showed that absence of FcγRIIB did not alter the ability of mouse MDSCs to mount a respiratory burst or produce iROS after PMA stimulation (Appendix D, Supplemental Figure 3). Next we tested the influence of CRP on wild type vs. FcγRIIB$^{-/-}$ cells. We found that human CRP enhancement of mouse MDSC generation at the expense of DCs was largely similar for FcγRIIB$^{-/-}$ compared to wild type (Figure 4A). However, the production of iROS by enriched MDSCs stimulated with human CRP (100 μg/ml) was significantly greater for FcγRIIB$^{-/-}$ than for wild type (Figure 4B). Despite this, human CRP did not enhance the ability of FcγRIIB$^{-/-}$ MDSCs to suppress mouse CD4$^{+}$ T cell proliferation (Figures 4C,D). These data suggest that CRP's ability to potentiate the immune suppressive actions of MDSCs is likely modulated both directly and indirectly by FcγRs.

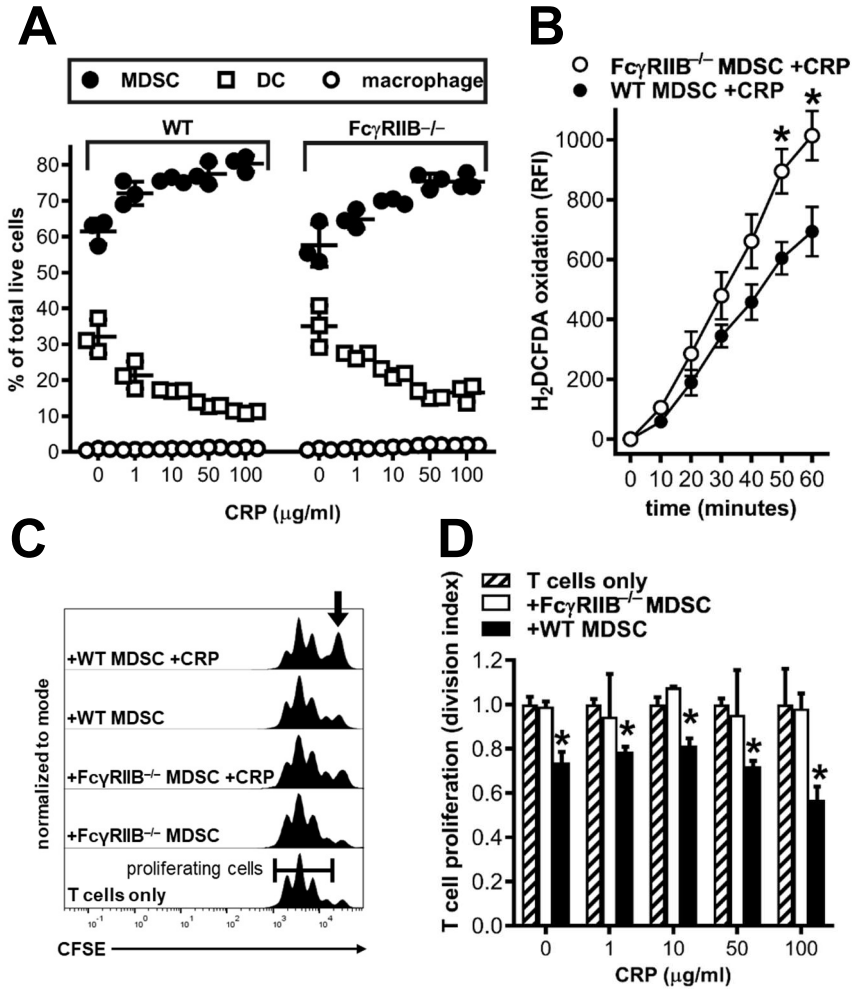


**Figure 4. Human CRP does not enhance the suppressive capacity of mouse FcγRIIB$^{-/-}$ MDSCs.** (A) Absence of FcγRIIB expression had no impact on human CRP's ability to drive MDSC generation in mouse BM cultures; representative mean ± SD of n = 4 conducted in triplicate (compare to Fig. 1 G). (B) In response to 100 μg/ml CRP, enriched mouse FcγRIIB$^{-/-}$ MDSCs exhibited enhanced production of iROS compared to WT. The asterisks indicate $p < 0.05$ (multiple one-tailed unpaired t-tests) for the representative mean ± SEM of 3 experiments conducted in triplicate. (C) CFSE dilution histograms showing that at a 1:5 E:T cell ratio, enriched mouse FcγRIIB$^{-/-}$ MDSCs were unable to suppress mouse CD4$^{+}$ T cell proliferation and this was not responsive to any dose of human CRP (1 – 100 μg/ml). (D) Division indices for the representative experiment shown in panel C. The asterisks indicate $p < 0.05$ compared to T cells only within CRP treatment (two-way ANOVA with Dunnett's multiple comparisons) for the representative mean + SD of 2 co-cultures conducted in triplicate.

*Human CRP Enables Primary Human Neutrophils to Suppress Proliferation of Autologous T Cells*

To ascertain the potential clinical relevance of our findings we sought evidence that human CRP also promotes an immune suppressive phenotype in human myeloid lineage cells. Because under certain conditions (such as cancer and severe injury) mature neutrophils can act as MDSCs (33), and because large numbers of them are easily obtained from the circulation, we isolated peripheral blood neutrophils from five healthy human donors for these studies. Like mouse MDSCs, human neutrophils treated with human CRP did not exhibit a respiratory burst (compare Figures 2A, 5A). Also like mouse MDSCs, human neutrophils treated with human CRP exhibited CRP dose-dependent production of iROS (compare Figures 2B, 5B).When co-cultured with autologous CD3/CD28 stimulated human $CD3^+$ T cells (1:1 E:T ratio) the neutrophils per se did not significantly impact T cell proliferation, but importantly in the presence of increasing amounts of human CRP they significantly suppressed it (Figures 5C,D). These data show that—like mouse MDSCs—human CRP grants suppressive capacity onto human primary blood neutrophils.

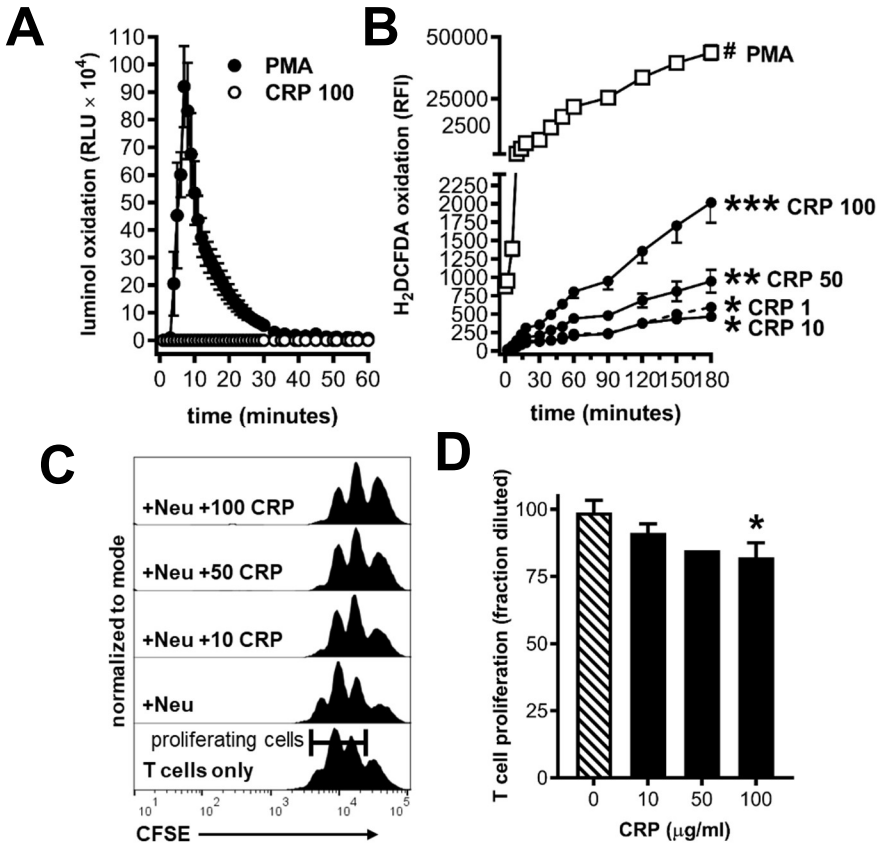


**Figure 5. Human CRP evokes a suppressive phenotype from human peripheral blood neutrophils.** Peripheral blood leukocytes were freshly isolated from healthy human donors (n = 5). (A) Robust ROS production (luminol oxidation) was achieved by human neutrophils when stimulated with 100 nM PMA but not with 100 μg/ml human CRP. (B) Intracellular ROS production ($H_2$DCFDA oxidation) by human neutrophils was significantly increased by human CRP in a dose-dependent manner. The asterisks compare untreated cells to CRP treatment (*$p$ < 0.05 at 60 min; **$p$ < 0.05 at 30 min; ***$p$ < 0.05 at 14 min) or to PMA treatment (#$p$ < 0.05 at 12 min) (two-way ANOVA with Dunnett's multiple comparisons) for the mean ± SEM. (C) Representative CFSE dilution histograms for autologous human CD3/CD28-stimulated T cells only (gray trace) or co-cultured with autologous neutrophils (Neu; 1:1 E:T), in the absence or presence of 10, 50, or 100 μg/ml human CRP. CRP bestowed suppressive function to the autologous neutrophils in a dose-dependent fashion. (D) The data are normalized to the maximum T cell proliferation achieved in the absence of neutrophils for the given CRP concentration. The asterisk indicates $p$ < 0.05 compared to co-cultures with 0 CRP (one-way ANOVA with Sidak's multiple comparisons) for the mean + SEM.

## Discussion

The genes encoding mouse and human CRP have very similar nucleotide sequences and genomic organization (34) and the mouse and human proteins share at least 70% amino acid sequence identity (35). The biological activity of mouse CRP has been the subject of comparatively few direct investigations [e.g., (36)], but because of this high homology it is generally assumed that mouse CRP has biological actions similar to that of human CRP (ability to activate complement, ability to bind FcRs, etc.). Importantly, because mouse CRP is not a major acute phase reactant (i.e., CRP concentration in the circulation of mice remaining below ~3 μg/ml under all conditions), CRPtg mice have been widely used to study the impact of the human CRP acute phase response in vivo. For example in our prior studies we established that the outcome of ischemic AKI is worse for CRPtg compared to wild type mice, linked this effect to the human CRP acute phase response and its associated heightening of renal infiltration by MDSCs during AKI (14, 15).

We also showed that the severity of AKI could be lessened by (i) reducing MDSC infiltration with an anti-Gr-1 antibody (15) or (ii) by targeting human CRP with an anti-sense oligonucleotide (37). These and other findings suggested that during AKI in mice, CRP promotes MDSC generation/expansion/renal infiltration and thereby propels the injury response; this effect is more pronounced in CRPtg because of their high levels of human CRP.

Expansion and infiltration of myeloid cells at the sites of injury and their acquisition of MDSC phenotypes and functions is well-described in the settings of cancer, trauma, and sepsis (16, 28). MDSC expansion is thought to be achieved by a shift in the hematopoietic pool toward granulocyte and monocyte progenitors (i.e., precursors

of neutrophils and monocytes/macrophages/DCs, respectively), thereby increasing the pool of effector myeloid cells available for quick resolution of the insult (38). Thus, during both acute (e.g., AKI) or chronic (e.g., cancer) pathological states, this response is thought to yield an expansion of myeloid-derived cells that exhibit a strong suppressor function: the so-called MDSC MDSCs are generally considered immature cells since they lack the nuclear morphologies and/or the portfolio of surface markers characteristic of mature neutrophils, macrophages, and DCs. Confounding this interpretation are reports that MDSCs can "mature" into neutrophils and macrophages within the same pathological milieu thought to promote their initial expansion (39, 40). These observations support the counterhypothesis that MDSCs are not a unique cell type derived from dedicated MDSC progenitors in the BM, but rather are mature leukocytes that acquire an atypical suppressive function in the periphery (16, 38). Notwithstanding this uncertainty and even in the absence of a specific marker for their identification and purification, bona fide MDSCs should have a T cell suppressive action.

Despite the present uncertainty about their true origin, identity, and developmental fate, we show herein that CRP can promote the expansion of mouse BM myeloid derived cells and enhance their suppressive phenotype. Furthermore, we show that exposure to CRP bestows upon mature human neutrophils a suppressive phenotype. The ability of CRP to promote MDSC expansion from mouse BM is likely related to CRP's ability to selectively increase the cell-cycling of MDSC progenitors, as CRP had no effect on the overall rate of cell death in BM cultures. Furthermore, even low doses of human CRP potentiated mouse BM progenitor commitment toward MDSCs and steered them away from DCs. These observations are in alignment with our recent report that

human CRP also inhibited the generation of mouse DCs in a completely different in vitro system (29). The ability of human CRP to promote mouse MDSC expansion on one hand, while inhibiting mouse DC expansion on the other, suggests that CRP might be a tonic regulator of BM progenitor lineage commitment and expansion—particularly during inflammation when the amount of human CRP is elevated. Likewise others have shown that human CRP can increase expression of CD206 (a marker of anti-inflammatory polarization) on monocytes, but not fully differentiated macrophages, also suggesting that CRP has more of an impact on less differentiated myeloid cells (41). In their sum, our latest findings suggest that (at least in mice) human CRP promotes the differentiation of myeloid progenitors into effector cells with suppressor functions, meanwhile dampening the development of myeloid cells that would otherwise promote adaptive immunity. Importantly, human CRP also evokes suppressive actions from human neutrophils.

Perhaps foreshadowing our findings by nearly 4 decades, Marcelletti et al. (42) reported that CRP potentiated monocytopoiesis by acting on FcR-expressing mouse myeloid progenitor cells in S-phase. FcRs are categorized based on their inhibitory or activating signaling potential and many of them are known to be utilized by CRP. Notably, the potent inhibitory FcR, FcγRIIB (CD32B), is used by CRP in both mice and humans (8, 29, 43–45). In this report we show that CRP does not rely on FcγRIIB to alter BM progenitor lineage commitment toward MDSCs. This outcome is similar to that reported by others, who showed that CRP can promote the generation of inflammatory macrophages from mouse BM even in the absence of FcγRs (46). Nevertheless, our results show that FcγRIIB is involved in CRP triggered ROS production by MDSCs, and CRP requires FcγRIIB to promote the suppressive function of MDSCs. Additional studies

are needed to fully explore the contribution of other FcRs that CRP might utilize, e.g., activating FcγRs (8) and the activating FcαR (30). The latter is of particular interest as recently it was shown that engagement of FcαR, as opposed to engagement of FcγR, more potently stimulates human neutrophils to kill cancer cells (47); CRP may be one of the FcR ligands mediating this effect. Additionally, further in depth research will be needed to thoroughly investigate the influence of CRP on myeloid lineage development.

One of the most potent suppressive mechanisms in the armamentarium of MDSCs is their ability to produce high amounts of ROS, whether derived from superoxide generated by membrane-bound NADPH-oxidases, the endoplasmic reticulum, or the mitochondrial electron transport chain. In our hands, CRP did not trigger a respiratory burst from mouse BM-MDSCs or human neutrophils, but did stimulate a monophasic increase in ROS consistent with their production of iROS. Furthermore, the production of iROS achieved statistical significance only when high concentrations of CRP were used, i.e., levels of CRP consistent with those found during inflammation (2). Although CRP stimulated a greater increase in iROS for FcγRIIB$^{-/-}$ than wild type mouse MDSCs (Figure 4B), CRP triggered iROS production by mouse MDSCs lacking all activating FcγRs (48) was not different than wild type MDSCs (data not shown). Taken together these findings are consistent with the notion that, during inflammatory episodes when CRP is elevated, CRP stimulates iROS production by mouse MDSCs and this is tempered by FcγRIIB engagement.

Despite being dispensable for CRP mediated enhancement of *in vitro* generation of MDSCs, FcγRIIB appears essential for CRP mediated promotion of their suppressive actions on T cells. To explain this seemingly paradoxical situation we are currently

investigating the possibility that conversion of superoxide to hydrogen peroxide, a cell permeant ROS, is impaired in FcγRIIB$^{-/-}$ MDSCs, perhaps due to decreased expression of antioxidants such as superoxide dismutase. Accordingly, in WT MDSCs the interaction of CRP with FcγRIIB might increase the expression of antioxidants, allowing for increased conversion of superoxide to hydrogen peroxide and thereby promoting the immune suppressive action of MDSCs. In the absence of FcγRIIB this pathway would be eliminated, allowing for accumulation of superoxide and other ROS but reduced conversion to hydrogen peroxide. In support of this model others have shown that superoxide-derived hydrogen peroxide generated by MDSCs is responsible for suppression of T cell activation and proliferation (49). Alternatively, CRP might regulate antioxidant gene expression by modulating the expression/action of the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is known to be highly expressed by MDSCs and thought to allow them to withstand the high oxidant stresses experienced during their expansion (50, 51). We are currently investigating this possibility. Also since M-MDSCs also produce RNS (52, 53), we are investigating whether CRP impacts MDSC generation of RNS.

The majority of renal MDSCs recovered from CRPtg mice subjected to AKI (15) and the majority of MDSCs generated in vitro from mouse BM precursors (this study) are of the PMN-MDSC subtype. Since the effects of human CRP on mouse MDSCs might be an aberration of a human protein/mouse cell system, and given the ongoing debate about PMN-MDSCs as a distinct lineage vs. neutrophils that gain suppressive functions in the periphery (33, 54), we sought to determine whether human CRP had the same effects on human neutrophils as it did on mouse MDSCs. Like mouse MDSCs, CRP-treated human

neutrophils did not exhibit a respiratory burst but did show a dose-dependent increase in their production of iROS. Most importantly, exposing human primary neutrophils to human CRP rendered them capable of suppressing the proliferation of autologous $CD3^+$ T cells. An important caveat is that in our suppression assays we used mouse $CD4^+$ T cells vs. human $CD3^+$ T cells (the latter comprised of 64.7 ± 3.8% $CD4^+$ cells and 28.7 ± 4.2% $CD8^+$ cells, n = 2 donors), so the two in vitro systems and the magnitude of the CRP effects therein cannot be compared directly. Nevertheless, the similarity in the effect of human CRP on mouse MDSCs vs. human neutrophils suggests that monitoring and targeting CRP might be a valid clinical strategy for overcoming MDSC/neutrophil mediated immune suppression. For example, CRP blood levels could be lowered using various available methods such as an antisense-oligonucleotide to CRP (37), small molecule inhibitors of CRP (55), or apheresis of CRP (56). Either of these CRP-lowering approaches might re-establish homeostatic hematopoiesis and/or foster the development of beneficial myeloid lineages. Consequently, patients with aberrant or over-represented pathologic myeloid effectors, such as those with cancer, AKI, etc., might benefit from specific lowering of CRP.

## Ethics Statement

This study was carried out in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals Eighth Edition* (NIH Academies Press, 2011), the Institutional Animal Care and Use Committees, and Institutional Review Board at UAB.

## Author Contributions

RJ and AS conceptualized this study, wrote the manuscript, conducted formal analysis, and data visualization. RJ, AS, and ZH reviewed and edited the manuscript. RJ, VK, AC, ZH, and AS designed experiments. RJ, VK, and AC conducted experiments.

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## Supplementary Material

The Supplementary Material for this article can be found in Appendix D, page 151.

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# MYELOID DERIVED CELLS WITH SUPPRESSOR FUNCTIONS IMPAIR RENAL TUBULAR EPITHELIAL CELL CYCLING

by

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Abstract

Acute kidney injury (AKI), the sudden impairment of kidney function, is characterized by renal epithelial cell death, impairment of renal filtration, and renal inflammation. What exactly constitutes the inflammation seen in AKI is not clear. Our laboratory previously addressed part of this issue showing that C-reactive protein, an acute phase reactant and mediator of immunity, exacerbates renal ischemia/reperfusion-induced AKI. We linked this effect to CRP's ability to increase the number of myeloid derived cells with suppressor functions (MDSC) in the injured kidneys. Herein we investigated whether the MDSCs per se have damaging effects on renal epithelial cells and further whether CRP could enhance this effect. We show that MDSCs inhibit the S phase cycling of primary renal tubular epithelial cells both with and without cell contact. We rule out several soluble mediators of this MDSC effect and discuss promising leads such as MDSC-produced monocyte-chemotactic protein -1 (MCP-1) and interleukin (IL)-10. These data suggest that MDSCs have a clear effect on renal epithelial cell cycling through a soluble mediator. CRP did not augment these MDSC functions but in an earlier report we showed that CRP potently promotes the generation of MDSCs from bone marrow hematopoietic progenitors. Thus, it is possible that following the renal insult, CRP greatly pushes the development of MDSCs that infiltrate the injury kidney, wherein they secrete MCP-1 to recruit additional effector cells and most importantly, inhibits renal epithelial proliferation. Targeted lowering of CRP would prevent this erroneous myeloid response and instead allow for recovery and repair from AKI.

## Introduction

Acute kidney injury (AKI) is the abrupt loss of renal filtration arising from a variety of etiologies. In ischemia/reperfusion-induced AKI, the initial hypoxic environment leads to acute tubular necrosis, obstruction of collecting ducts and/or renal vasculature, and inflammation-mediated immune responses. The early proximal tubule cells are particularly susceptible to ischemic-induced cell death, often leading to loss of their brush border and disruption of the epithelial basement membrane which in turn allows for filtrate back leak, sloughing of dead and viable cells, and eventual cast formation (1). Although the definition of inflammation in AKI remains equivocal, it is known that AKI is accompanied by the activation of complement, the production of chemokines, and the induction of the acute phase response (2). Subsequently, kidney-resident immune cells are activated that promote the rapid influx of circulating leukocytes (2) potentiating emergency myelopoiesis: a shift towards greater numbers of common myeloid progenitors. The combination of compromised renal epithelial and endothelial architecture, dead cells, debris, and hyper-infiltration of immune cells can culminate in renal blood flow and filtrate obstruction (3). Together with persistent inflammatory signals and impaired renal stromal cell regeneration results in ineffective resolution of the injury. As a consequence, patients often develop chronic kidney disease, end-stage renal disease, and eventual death.

Clinically, the measurement of C-reactive protein (CRP) levels is used to assess the magnitude of the inflammation in settings of both acute and chronic renal insufficiency. CRP is the prototypical acute phase reactant meaning that upon sufficient systemic inflammation, hepatic production of CRP increases from baseline (<1 μg/ml in the blood) to 100-fold or more within hours (4,5). From these first observations in

humans, it was noted that these high CRP levels persisted as long as the extent of the injury; no doubt similar to those patients with high levels of CRP with their AKI that progresses chronic kidney disease.

Our laboratory thus set out to determine whether elevated CRP levels found in kidney disease patients, particularly those with AKI, is causal or merely correlates with the inflammatory processes. To this end, we used the preclinical model of AKI of surgical bilateral renal ischemia/reperfusion in our CRP transgenic mice (CRPtg) and CRP knockout mice. As expected, the CRPtg mice have worsened AKI as measured by serum creatinine, urine albumin, and kidney histological assessment, as compared to wild type and CRP knockout mice (6,7). In the histological viewings, it was noted that in CRPtg kidneys, compared to wild type, there was an overabundance of infiltration of CD11b+ Gr-1+ cells with the ability to suppress the proliferation of CD3/CD28 stimulated T cells, also known as myeloid derived cells with suppressor functions (MDSC) (6,7).

MDSCs are widely studied in the tumor setting where they prevent anti-tumor responses largely through generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and amino acid starvation (arginase and indoleamine 2,3-dioxygenase) that experimentally can be measured through their ability to suppress the proliferation of T cells (8-10). MSDC ontogeny is under debate; however there is consensus that MDSCs arise during emergency myelopoiesis and they maintain an immature state permitting their acquisition of suppressive functions from pathological signals (11).

The increased numbers of MDSCs in the injured CRPtg kidneys was prevented by the ablation of CRP expression, resulting in a decrease in the severity of AKI (6). This

clear control of MDSC mobilization to the injured kidney by CRP lead us to determine whether CRP impacted MDSCs directly. Previously, we showed that CRP increases the generation of MDSCs from bone marrow progenitors, dose-dependently increase their intracellular ROS generation, and enhances the suppressive activity of MDSCs on T cell proliferation (12).

We sought here to determine whether bone marrow derived MDSCs could impair primary renal tubular epithelial cells (RTEC), likely through their ability to produce ROS. Furthermore, we tested whether CRP could stimulate or enhance any harmful effects the MDSCs might display. We report herein that RTEC cycling through S phase is impaired by MDSCs in co-cultures without and with direct cell contact. These data suggested that an MDSC soluble mediator is involved which led us to assess the involvement of ROS, RNS, and/or cytokines. Surprisingly, quenching of ROS with catalase had no effect on MDSC suppression of RTEC cell cycling, nor did treatment with CRP stimulate an enhance MDSC effect. As these MDSCs produced no detectable RNS, their suppressive mediator may be a cytokine and further studies are needed to identify which cytokine. However, we show that MDSCs potently express monocyte chemoattractant protein-1 (MCP-1, CCL2) which may be involved in their heightened accumulation in CRPtg injured kidneys.

*Primary Renal Tubular Epithelial Cell Culture*

Both kidneys from one mouse were removed, submerged in RTEC media, mechanically homogenized with a clean razorblade, and the resultant slurry passed through a 70 µm cell strainer. After centrifugation, the cell pellet was seeded onto a 10 cm diameter plate coated with 5 µg/cm$^2$ collagen type I from rat tail (Corning 354236) in RTEC media (renal epithelial cell growth medium 2, Promocell C-21630) supplemented with 1X antibiotic/antimycotic (Gibco 15240062), incubated overnight at 37°C, 5% $CO_2$ until the next day when the non-adherent cells were collected and re-seeded onto a fresh collagen coated plate. Media was changed after 72 h. After 7 d in culture, the RTEC were used in subsequent assays or passaged. For passage, RTECs were gently lifted from the culture plate surface with a cell scraper in a continuous swipes, gently pipetted to homogenize in fresh media, and seeded onto new collagen coated plates.

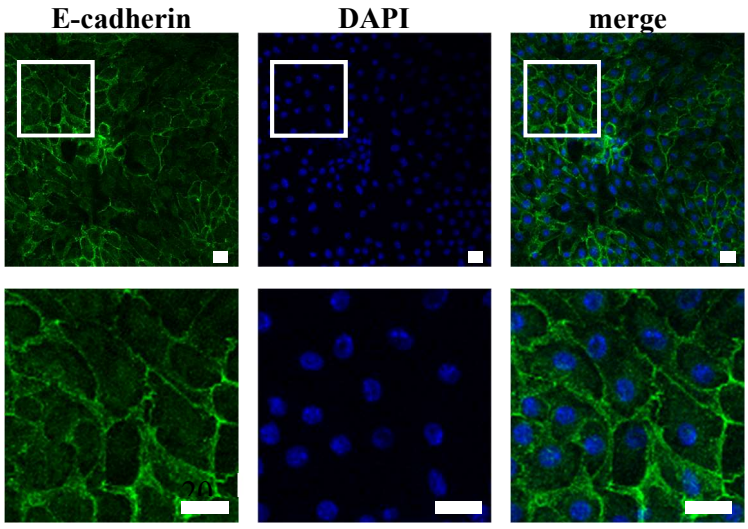


**Figure 1.Primary renal tubular epithelial cells.** Mouse kidney cells were used to expand primary RTECs. After 7 d in culture, the cells show a characteristic cobblestone appearance and stain for E-cadherin (z-stacked confocal immunofluorescence). Bars denote 20 µm.

*Immunohistochemistry and Confocal Microscopy*

RTEC morphology and E-cadherin expression was confirmed by immunofluorescent microscopy (Figure 1). Briefly, RTECs were expanded on a glass coverslip coated in collagen for 7 d. The adherent cells were washed and fixed in ice-cold 100% methanol for 5 min at -20°C. The monolayer was then blocked with 5% BSA (in $dH_2O$, 0.2 μm PES filtered, GE #6780-2502) for 60 min at RT. Next, the cells were probed with rabbit anti-mouse E-cadherin monoclonal antibody (clone 24E10, Cell Signaling Technologies 3195S) overnight at 4°C. The next day, untreated cells and primary antibody probes were incubated with a polyclonal, cross-absorbed $F(ab)_2$ goat anti-rabbit IgG (H+L) conjugated to Alexa Fluor 488 (Invitrogen A11008) for 1 h at RT. Slides received one drop of either prolong gold anti-fade mountant with DAPI (Invitrogen P36935) or mounting media for fluorescent microscopy (KPL 71-00-16). The coverslips were lifted out of the culture plate, placed face down into the mounting media, and cured overnight at RT in the dark, after which the coverslips were then sealed. The slides were either stored at 4°C in the dark or visualized on a Nikon A1 confocal microscope with NIS Elements AR 5.02.00. Each channel was calibrated with a slide stained for DAPI alone, secondary antibody alone, or left unstained. Images were z-stacked by beginning image acquisition at the bottom slice of the monolayer and z-stepping to the top of the layer (~2 μm total) and rendered to create a 2D image.

*Bone Marrow Myeloid Derived Suppressor Cell Culture*

Primary bone marrow (BM) myeloid derived suppressor cells (MDSC) were generated as previously described [152]. Briefly, BM from wild type mice was flushed from the long-bones of the hind legs, strained, the red blood cells were lysed, and the resulting

suspension seeded at $1 \times 10^6$ cells/ml in Eagle minimum essential medium, alpha-modification (Lonza 12-169F) supplemented with 10% heat inactivated-fetal bovine serum (Gibco 10082147), 2 mM GlutaMAX (Gibco 35050061), 100U per ml/100 μg per ml penicillin/streptomycin (Gibco 15140122), 1 mM sodium pyruvate (Gibco 11360070), 55 μM β-mercaptoethanol (Gibco 21985023), and 40 ng/ml mouse granulocyte/ macrophage-colony stimulating factor (GM-CSF; Shenandoah Biotechnology 200-15). Cells were incubated at 37°C, 5% CO2 for 4 d. The cell culture was harvested and negatively enriched using the EasySep mouse CD11c positive selection kit II (StemCell Technologies 18780) according to the manufacturer's instructions. This yielded highly purified (94 ± 1.5%) CD11c– enriched MDSCs (hereafter, referred to as MDSC) (12).

*RTEC:MDSC co-cultures*

For co-cultures with MDSC:RTEC contact, $1 \times 10^6$ MDSCs were seeded into one well of a 12-well plate with an RTEC monolayer. The cells were co-cultured for 18 h and 20 μM bromodeoxyuridine (BrdU; Sigma B5002) was added 2 – 4 h prior to harvesting. The non-adherent cells (including dead RTEC and MDSCs) were discarded and the adherent RTECs were assessed by flow cytometry.

For co-cultures without MDSC:RTEC contact, $1 \times 10^6$ MDSCs were seeded into a 0.4 μm, PET transwell (Corning 353494, 353090) above a RTEC monolayer. Note the 0.4 μm pore size precludes any cell migration but allows fluid exchange and small molecule diffusion. As indicated, 1 – 100 μg/ml of highly purified human CRP (US Biological Sciences C7907-26A) was added to the MDSCs in the transwell insert. The cells were then co-cultured for 18 – 24 h and BrdU was added to the entire culture medium 3 h prior to harvesting the RTEC monolayer. Following co-culture, the

monolayers were assessed by flow cytometry for either BrdU incorporation or Annexin V binding as described below.

*Bromodeoxyuridine incorporation*

RTECs were fixed with cold 70% ethanol, permeabilized with $Na_2B_4O_7$, CD16/CD32 blocked, and the incorporated BrdU was probed with an APC conjugated anti-BrdU antibody (clone Bu20a, BioLegend 339808). Immediately before analysis, 1 µg of 7-aminoactinomycin D (7-AAD, Invitrogen A1310) was added. Cell cytometry was performed on a BD LSRII cytometer using BD FACSDiva version 6.1.3 software, a standard gating strategy was used to identify cells in the G0/G1, S, and G2/M phases of cell division as analyzed with FlowJo version 10.3. Single cells were gated on using an SSC-A × SSC-H dot plot, and apoptotic (7-AAD$^{lo}$) cells were excluded. Untreated live cells were used to gate G0/G1 (7-AAD$^{int}$BrdU$^{lo}$), S (7-AAD$^{lo-hi}$BrdU$^{+}$), and G2/M (7-AAD$^{hi}$BrdU$^{+}$) phases of cell division. S phase was further subdivided into early (7-AAD$^{lo}$BrdU$^{+}$) and late S phase (7-AAD$^{hi}$BrdU$^{+}$).

*Annexin V binding for detection of apoptosis*

After transwell co-culture, the RTEC monolayers were harvested and assessed for apoptosis according to the manufacturer's protocol for the Annexin V Apoptosis Detection Kit (BioLegend 640914). Briefly, cells were assayed in Annexin V binding buffer and stained with FITC conjugated Annexin V and propidium iodide solution for 15 min at RT. The cells were immediately assayed on a BD LSRII cytometer using BD FACSDiva version 6.1.3 software and analyzed using FlowJo version 10.3. Represent-

ative gating strategy is shown in Appendix E, Supplemental Figure 1. and the frequency (%) apoptotic includes those cells in early and late apoptosis.

*ELISA for assessment of cytokines*

MDSCs were grown and enriched as described above and $1\times10^6$ MDSCs/ml were added to a 12-well plate in MDSC media without GM-CSF added. The MDSCs were treated in triplicate with 1 μg/ml LPS serotype O55:B5 (Sigma L2880), or with 100 μg/ml human CRP, or with 0.797 μM mouse serum-purified IgG (Sigma I5381), the latter an equal molar amount to 100 μg/ml human CRP. After 24 h, the well contents were spun down to remove cells and the supernatants collected and used in enzyme-linked immune-sorbent assays (ELISA) according to the manufacturer's recommendations. The cytokines assessed were interleukin (IL)-10 (Invitrogen 88-7105), monocyte chemoattractant pro-tein-1 (CCL2; Invitrogen 88-7391), transforming growth factor-beta 1 (TGF-β1; Invitro-gen 88-8350), interferon (IFN) gamma (IFNγ; Invitrogen 88-7314), IFNα (Invitrogen BMS6027), and IFNβ (Invitrogen 424001). Culture supernatants from each treatment triplicate were run in duplicate; data reported are thus the average of 6 replicates.

*Statistical Analyses*

The data were averaged and are reported as the mean ± SEM. ELISA data are from one culture conducted in 6 technical replicates and are reported as the mean ± standard deviation (SD). One-tailed paired Student's t-tests were conducted to compare the mean of replicates from individual experiments of the RTECs without MDSCs to the paired RTECs co-cultured in the presence of MDSCs (either with contact, Figure 2C and D, or without contact, in transwells Figure 3B and C). A repeated measures one-way

analysis of variation (ANOVA) was conducted comparing within experiments for the mean of triplicates from RTECs co-cultured with MDSCs (that were untreated or treated with 100 μg/ml CRP or 50 U/ml catalase) normalized to the respective treatment control RTECs (that were untreated or treated with 100 μg/ml CRP or 50 U/ml catalase). ELISA data was tested with a one-way ANOVA compared to untreated. Statistical analyses were conducted in GraphPad Prism 8.

## Results

### *MDSC inhibit RTEC S phase cycling*

We previously established that i) MSDCs infiltrate injured kidneys and ii) primary MDSCs generated from mouse bone marrow strongly inhibit the proliferation of CD3/CD28 stimulated T cells [152, 153]. We sought here to determine whether MDSCs have similar deleterious actions on primary renal tubular epithelial cells (RTEC; Figure 1). To test this, primary RTECs were co-cultured with $1 \times 10^6$ MDSCs in direct contact for 18 h. To assess the effect of MDSCs on RTEC cell cycling, the RTECs in S phase were analyzed by flow cytometry for their incorporation of BrdU (Figure 2). While the presence of MDSCs had no effect on the proportion of RTECs in S phase (Figure 2C), they did affect their rate of transit through S phase, i.e. in the presence of MDSCs fewer RTECs were in early S phase compared to late S phase (Figure 2D). These data are consistent with MDSCs impairing the ability of RTECs to exit S phase as compared to the rate exemplified by RTECs alone.

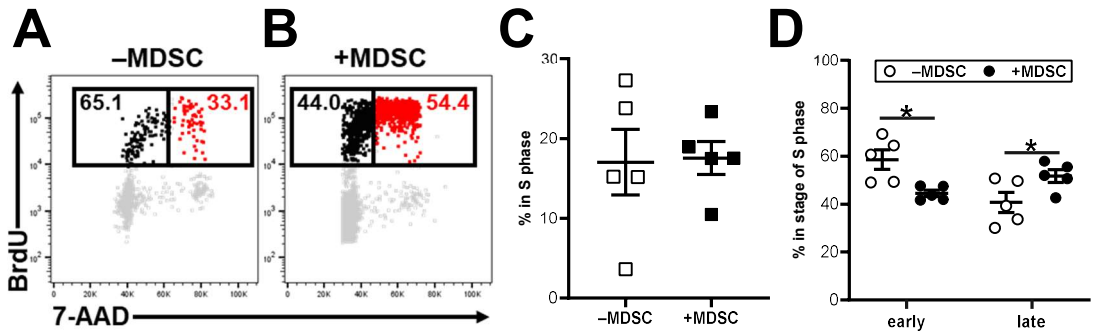


**Figure 2. MDSCs block RTEC exit from S phase.** Primary RTECs were expanded for 7 d and then co-cultured with $1\times10^6$ MDSCs. BrdU was present during the last 2 – 4 h of the 18 h co-culture and the adherent $BrdU^+$ RTECs (those in S phase) were analyzed by flow cytometry. RTECs alone (A) or RTECs co-cultured with MDSCs (B) gating strategy for early (black) or late (red) S phase. (C) The proportion of RTEC in S phase was not affected by the presence of MDSCs (■). (D) RTEC kinetics through S phase was significantly affect by MDSC action (●); replicate mean ± SEM cultures (n = 5) from 3 experiments analyzed by two-tailed ratios paired t-test (*).

A key feature of MDSCs is their ability to suppress nearby cells through soluble mediators such as inhibitory cytokines, reactive nitrogen species (RNS), and primarily through reactive oxygen species (ROS) (9). As a consequence, MDSCs do not need cell:cell contact to affect bystanders. Therefore, to determine whether MDSCs produce a soluble mediator that impairs RTEC, RTEC monolayers co-cultured below a transwell insert containing $1 \times 10^6$ MDSCs (Figure 3A). The pores of the transwell membrane (0.4 μm) are allow small molecules to pass through but prevents MDSC migration. In the absence of physical contact with RTECs, the MDSCs were still able to inhibit the ability of RTECs to enter S phase (Figure 3B). Interestingly, the MDSCs did not alter the proportion of RTECs undergoing apoptosis, as assessed by Annexin V staining (Figure 3C). Together, these data suggest that MDSCs affect RTEC cycling, and thus their proliferation, but not to their programmed cell death even in the absence of contact.

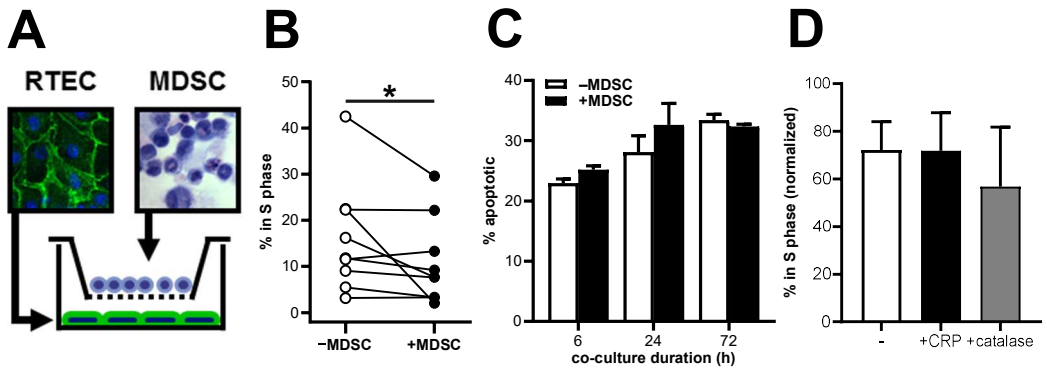


**Figure 3. MDSCs impede RTEC entry into S phase.**(A) RTECs (○) were grown and a transwell insert with 1 × $10^6$ MDSCs (●) was added for 18 – 24 h. BrdU was added 3 h prior to the end of the co-culture and S phase analyzed as in Figure 2. (B) Each line compares the mean of replicates from an experiment (n = 9) and significance (*) was determined by a one-tailed paired t-test. (C) Regardless of co-culture duration (6 – 72 h), MDSCs (black bars) did not affect the apoptotic frequency of RTECs visualized by flow cytometric Annexin V staining; mean + SEM of two experiments. (D) Treatment with 100 μg/ml human CRP (black bar) or 50 U/ml catalase (gray bar) did not alter MDSC inhibition of RTEC entry into S phase (compare to the effect of untreated MDSCs, white bar). Data are normalized to treatment control RTECs without MDSC exposure; the replicate mean + SEM of two experiments.

*The soluble mediator of MDSC action*

We previously determined that i) CRP enhances the suppressive action of MDSCs on T cell proliferation and ii) CRP increases the intracellular ROS generation of MDSCs (12). To test whether CRP signals MDSCs to increase their noxious effects on RTECs via their production of ROS, human CRP (100 μg/ml) or catalase (50 U/ml) was added directly onto the MDSCs in the transwell in co-cultures (Figure 3D). These concentrations were used because RTEC S phase is not affected by catalase alone (data not shown) or by CRP alone (Appendix E, Supplemental Figure 2.). The ability of MDSCs to impair RTEC S phase cycling was not affected by CRP or catalase treatment. Thus, it is unlikely that MDSC produced ROS is responsible for their ability to inhibit RTEC entry into S phase.

Other methods of MDSC suppression were explored. As MDSCs are reported to produce RNS, we tested the culture supernatants of primary MDSCs for nitrate via Griess

assay. After stimulation with 1 μg/ml LPS, 0.8 μM IgG, or 100 μg/ml CRP (alone or in conjunction with PMA), there was no detectable amount of nitrate (data not shown). We therefore turned our attention to cytokine production by MDSCs (Table 1). Untreated MDSCs produced detectable amounts of MCP-1 (CCL2) and IFNα. Interestingly, LPS stimulation significantly increased MDSC production of MCP-1 and IL-10. Stimulation with heat-aggregated IgG or CRP had no effect. Whether these molecules are involved in their suppressive function remains to be explored. These data are interesting for future AKI studies where inflammatory TLR4 signaling may be participant (13) and act synergistically with CRP's ability to potently increase MDSC intracellular ROS.

**Table 1. MDSC cytokine production after 24 h stimulation.**

| | IL-10 (pg/ml) *mean ± SD* | MCP-1 (ng/ml) *mean ± SD* | TGF-β1 (ng/ml) *mean ± SD* | IFNγ (pg/ml) *mean ± SD* | IFNβ (pg/ml) *mean ± SD* | IFNα (pg/ml) *mean ± SD* |
|---|---|---|---|---|---|---|
| untreated | ND | 117.1 ± 174.1 | 0.16 ± 0.01 | ND | ND | 14.8 ± 8.6 |
| 100 µg/ml CRP | ND | 321.1 ± 7.3 | 0.19 ± 0.03 | ND | ND | 13.5 ± 3.0 |
| 0.8 µM IgG* | ND | 355.8 ± 29.0 | 0.29 ± 0.15 | ND | ND | — |
| 1 µg/ml LPS | 2243 ± 75 | 643.6 ± 47.9# | 0.16 ± 0.08 | ND | ND | — |
| media | ND | ND | 0.28 ± 0.00 | ND | ND | — |
| one-way ANOVA | — | #$p = 0.0035$ | ns | — | — | ns |

heat-aggregated (*); not detected (ND); not tested (—); not significant (ns)

## Discussion

Previously, we established that CRPtg mice experiencing AKI have increased infiltration of CD11b+ Gr-1+ cells with suppressive functions (i.e. MDSCs), compared to wild type mice, and that ablation of CRP expression prevented their accumulation in the injured kidney with a concomitant decrease in kidney damage (6). Could these MDSCs be the executors of damage beset by CRP? To answer this question, we sought herein to further our understanding of CRP's role on myeloid cell functions and their consequences on renal epithelia. We showed that MDSCs can impair the cell cycling ability of RTECs, when co-cultured in the presence or absence of contact. MDSCs in direct contact with the RTECs did not alter the proportion of RTECs in S phase but did impair their ability to proceed normally through S phase. Intriguingly in the absence of contact, MDSCs impaired the overall ability of RTECs to enter S phase. The MDSC effect on RTECs is slight and did not affect their rate of programmed cell death, indicating that RTECs are stalling at cell cycle checkpoints and will need to be explored further. Transwell co-culture data suggest that the MDSCs produce a soluble mediator. MDSCs are known to toxically produce ROS and RTECs are prone to ROS-mediated damage, particularly in ischemic-AKI. Thus, we sought to determine whether quenching H2O2, the stable extra-cellular byproduct of ROS, with catalase would alleviate the MDSC effect. However, breakdown of H2O2 did not prevent MDSC inhibition of RTEC S phase entry.

Based on our previous studies, we showed that CRP significantly increases MDSC i) inhibition of T cell proliferation and ii) intracellular ROS. We therefore sought to test whether CRP could enhance the MDSC suppression of RTECs. However, addition of CRP had no impact on this MDSC effect. Furthermore, we rule out ROS, RNS and some cytokine production as potential soluble mediators of MDSC action. This report

demonstrates that CRP had no effect on MDSC functions in these experimental conditions.

As an alternative explanation for CRP's deleterious functions post-AKI in CRPtg mice we also tested the ability of CRP to inhibit RTEC cell cycling. In our hands, human CRP per se had no effect on the cell cycling of mouse primary RTECs generated in this study. These data conflict with a previous report that human CRP impairs the S phase cycling (BrdU incorporation) of HK-2 human renal epithelial cell line (14). Whether this reflects a real difference in CRP biological activity or a simple human versus mouse incompatibility is not known.

It is possible that the small secretion of IFNα by MDSCs is responsible for their inhibitory effect on RTECs. A previous study showed that IFNα can lead to barrier destabilization of the renal proximal tubule epithelium and their eventual apoptosis (15). Another study showed that AKI is exacerbated by plasmacytoid dendritic cell IFNα production, which they linked to its targeting of RTEC apoptosis (16). However, in the in vitro assays conducted in this study the MDSC production of IFNα seems unlikely to be the probable soluble mediator.

Of the tested cytokines, MDSC baseline expression was highest for MCP-1 (CCL2). Whether MCP-1 is involved in the inhibitory effect of MDSCs in this RTEC co-culture setting remains to be explored. It is known that renal cell types can express MCP-1 and this expression is increased during experimental models of AKI. Early after AKI, MCP-1 expression is predominately by renal cells and is later mainly produced by the then infiltrated monocytes/macrophages (17,18). Experimental studies further showed that MCP-1 can be detected in the urine post-AKI, and can also be found in humans post-

AKI (19,20). Our data adds to these findings in that post-AKI MDSCs may also secrete MCP-1, leading to an unfortunate feedforward recruitment of MDSCs into the injured kidney. However, caution must be taken to completely abolishing MCP-1 mediated recruitment of myeloid cells to the injured kidney. In a renal ischemia/reperfusion mouse model, whole-body knockout of MCP-1 led to normal monocyte recruitment to the kidney yet the mice had severe kidney damage and overall increased mortality (21). Thus, a balance is needed to recruit immune cells into the kidney to dampen the acute response but allow enough inflammation to permit control of the injury response.

Our previous work showed that CRP increases the expansion of MDSCs from BM progenitors (12) and in the CRPtg mouse there is increased numbers of MDSCs found in the injured kidney (7). It is possible that in the CRPtg mouse, their heightened MDSC renal infiltration is due to i) CRP increasing their generation and ii) MDSCs per se express MCP-1 to recruit their kin to the kidney. In other words, during the acute phase response CRP functions as a programmer for myeloid cells with suppressor functions (MDSC) which then infiltrate the injured kidney in response to renal production of MCP-1. This initial production of MCP-1 is needed for sufficient control of the inflammatory response to the injury per se (21). However, in the CRPtg mice, it may be that their distribution of hematopoietic progenitors is skewed even further by CRP to develop more MDSCs or CRP coopts more myeloid cells into gaining suppressive functions. Within this study, the MDSCs did not exhibit potent inhibitory effects on the RTECs. However, in vivo in CRPtg mice (and humans) there would be simply be more MDSCs yielding more cells that could cause physical blockage of renal blood flow or the filtrate. Alternatively or in addition, in CRPtg post-AKI kidneys, the MDSCs receive additional

pro-inflammatory signals thereby bolstering their deleterious effects. For example, we showed that MDSCs robustly produces IL-10 upon TLR4 stimulation (LPS). By secreting an anti-inflammatory molecule (e.g. IL-10), the MDSCs may dampen the local renal inflammatory response but as a consequence they are impeding productive healing responses, i.e. macrophage efferocytosis. Again, this fine balance of immunity is critical to prevent maladaptive repair and subsequent kidney disease. An imbalance of CRP promoting MDSCs can be seen in a model of progressive kidney disease wherein CRPtg mice had increased MCP-1 expression, and increased macrophage infiltration, and overall worse fibrosis (22).

We propose that CRP is an easily quantifiable 'cytokine' that drives myelopoiesis to produce suppressive myeloid cells (i.e. MDSCs) that in turn infiltrate the injured kidney and prevents sufficient inflammatory processes and healing. Therefore, ablation of CRP post-AKI would restore the balance of myeloid cells towards differentiated cells that better resolve the injury (23). This rebalancing of the myeloid progenitors' programming would therefore prevent the exaggerated pathology caused by high CRP levels and the progression to chronic kidney disease.

## Ethics Statement

This study was carried out in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals Eighth Edition* (NIH Academies Press, 2011), the Institutional Animal Care and Use Committees, and Institutional Review Board at UAB.

## Author Contributions

RJ and CL conducted the experiments. RJ and AS conceptualized this study and its experiments. RJ wrote the manuscript, conducted formal analysis, and data visualization. RJ and AS edited the manuscript.

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## Supplementary Material

The Supplementary Material for this article can be found in Appendix E, page 155.

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# DISCUSSION

C-reactive protein is an ancient pattern recognition molecule that is a soluble adapter molecule mediating reactions between innate and adaptive immunity. This dissertation project focused on the ability of CRP to potentiate myeloid lineage cells with its implications for renal ischemia reperfusion injury and beyond. I will discuss i) the role of CRP in potentiating myelopoiesis, ii) the effect of CRP on mature myeloid cells, iii) the role of CRP in autoimmunity, iv) the CRP in AKI, and finally, v) the potential role of CRP in neoplasms. Overall, the main theme is that CRP is a *tonic suppressor of immunity* by instructing a regulatory program on i) the development of myeloid cells from progenitors and ii) directly on differential myeloid cell types. Additionally, CRP can dampen the immune response with ligand binding functions (not experimentally explored in this project, but its relevance summarized below). The functional consequence of these CRP-programmed cells and the ligand-binding actions of CRP is manifest in autoimmunity, AKI, and likely cancer.

## CRP Instructs a Regulatory Myeloid Cell Phenotype

The chapters *C-reactive protein impairs dendritic cell development, maturation, and function: implications for peripheral tolerance* and *C-reactive protein promotes the expansion of myeloid derived cells with suppressor functions* explored the role of CRP on potentiating the *in vitro* bone marrow (BM) generation of dendritic cells (DC) and myeloid derived cells with suppressor functions (MDSC), respectively. In both reports

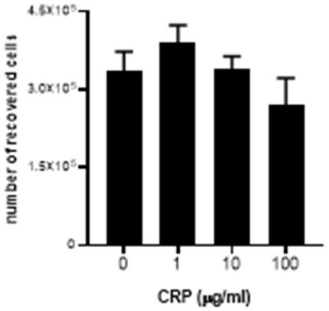


**Figure 1. CRP does not significantly alter the number of recovered cells following BM-MDSC culture.** BM-MDSCs cultured with increasing concentrations of human CRP showed no difference in the total number of cells output by the BM progenitors after 4 d in culture. Numbers were back-calculated from flow cytometry flow rate on an Attune NxT. Mean + SD of triplicates from one experiment; not significant by a one-way ANOVA compared to 0 CRP.

we showed that CRP tips the lineage commitment of BM precursors away from dendritic cells (DC). Furthermore, in culture conditions meant to expand MDSCs, as little as 1 µg/ml CRP increased the proportion of MDSCs and at the same time decreased the $CD11c^+$ DCs found within the same culture. CRP did not increase the overall rate of apoptosis nor necrosis in these BM cultures but did increase the cycling rate through S phase. Furthermore, CRP does not necessarily preclude the ability of progenitors to respond to colony stimulating factors (CSF) because there is still development and equal output of the number of cells from the culture, even with pathological concentrations of CRP (i.e. 100 µg/ml; Figure 1). These data suggest that CRP modulates the BM yield and not a mass culling of a progenitor pool or its progeny.

These observations are not necessarily new: previous reports showed that CRP modulates monocytic/macrophage progenitors[154-156]. CRP was shown to potently inhibit (by 30 – 60%) the colony formation of BM cells into macrophages yet did not have an effect on granulocytes. This action of CRP is likely mediated through receptor (i.e. binding with its A face), as CRP complexed with phosphocholine was able to have modulatory functions. Furthermore, the BM cells susceptible to CRP inhibition were in S phase, expressed Fc receptors, and still produced macrophage-CSF (M-CSF). Absent from these studies is the effective range of CRP and also were a decade before the discovery of the receptors CRP bound (FcγRs). Regardless, it is evident that CRP skewed

the cellular composition of myeloid development away from macrophages with no inhibitory effect on the granulocyte pool.

These historical data and my new data show that CRP retards BM progenitor differentiation into DCs and macrophages but not into granulocytes. I propose that CRP instructs a myeloid development program that yield cells that are suppressive, regulatory, et cetera (Figure 2). There is a growing consensus that G-MDSCs and M-MDSCs simply represent a phenotype of neutrophils and monocytes, respectively. I propose that CRP is a fluid phase mediator that drives this phenotype program. Specifically, I believe that CRP drives this regulatory program at two distinct steps: first, by influencing *de novo* cell type generation from BM and second, by directly modulating differentiated myeloid cell function. I will discuss the first hypothesis in this section and second in the next section.

For the purposes of this discussion, it is acknowledged that myelopoiesis does not necessarily follow a strict hierarchy as depicted, whether it is in the BM or in the periphery, and whether it is during "health" or disease. Furthermore, there are reports of reprogramming myeloid cells in the periphery, suggesting that 'differentiation' is a fickle term. Herein, I use the term 'differentiation' to mean a myeloid cell that has limited self-renewal capacity (compared to a progenitor) and has largely become DC, macrophage, neutrophil, etc. Precluded from this discussion is the involvement of fetal-seeded myeloid cells into the periphery and their capacity to serve as self-renewing precursors and contributors to immunity.

It is unlikely that CRP simply stops myelopoiesis, as i) MPs were still able to produce M-CSF, ii) granulocyte formation was not outright inhibited by CRP [156], and iii) CRP does not alter the output of BM cultures (Figure 1). Instead, CRP is programing

progenitor differentiation towards producing regulatory myeloid cells. This mechanism is likely weak, but is still operant, during health where baseline CRP levels are low. However, during acute phase settings, or in settings of chronic immune reactions, higher CRP levels increasingly drives this regulatory myeloid development program.

Each differentiated myeloid cell type is affected by CRP to varying degrees. Based on my *in vitro* observations it appears that CRP potently and robustly dampens the differentiation into DCs. This may be due to their 'natural' ability to promote adaptive immune response and to achieve a regulatory phenotype requires secondary signals with the periphery. The studies of CRP's effects on monocyte progenitors showed that their development into macrophages was inhibited, based on morphological observation;

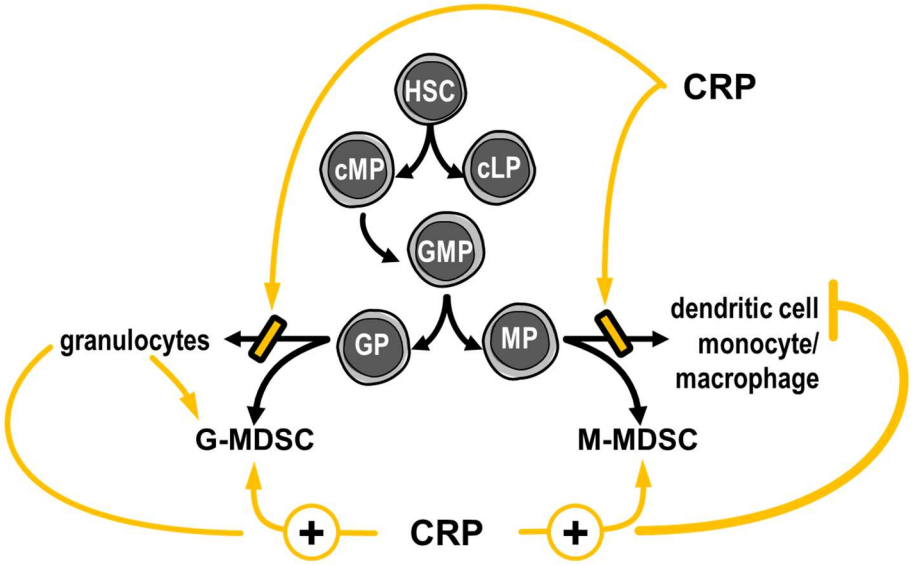


**Figure 2. CRP instructs a regulatory program during myelopoiesis and directly on differentiated myeloid cells.** I propose that CRP during myelopoiesis (yellow notches) i) potently inhibits DC differentiation, ii) prevents the formation into macrophages, iii) filters the generation of neutrophils, thereby pushing towards 'MDSC' generation. This mechanism would be proportional to the amount of CRP in sera: during baseline, CRP promotes a small proportion of MDSC generation and high CRP levels programs myelopoiesis to predominately produce MDSCs. Based on *in vitro* data, CRP does not change the proportion of granulocytic (G-) or monocytic (M) MDSC populations output by the BM pool. Separately, the direct effects of CRP on 'differentiated' myeloid cells in the periphery potently inhibits DC functions, augments MDSC functions, and possibly potentiates granulocytes towards G-MDSCs. HSC: hematopoietic stem cell; cLP: common lymphoid progenitor; cMP: common myeloid progenitor; GMP: granulocyte/monocyte progenitor; MP: monocyte progenitor; GP: granulocyte progenitor.

today, we know that MDSCs include a monocytic subtype (M-MDSC) that express the same surface markers as monocytes but have different functions and morphology. It is possible that CRP prevents the development into the morphology resembling macrophages and instead pushes their phenotype towards M-MDSCs. I believe that CRP influences neutrophils and granulocytic MDSCs (G-MDSC) the least. Discussed below is evidence that at baseline, CRP diminishes the frequency of neutrophils generated. Additionally, I showed that CRP increases the frequency of $CD11c^{-}F4/80^{-}CD11b^{+}Gr\text{-}1^{+}$ cells generated from BM, however within this population of MDSCs, CRP did not skew the proportion of G- to M-MDSCs. This equal effect of CRP on both the GP and MP production of regulatory neutrophils (G-MDSC) and monocytes (M-MDSC), respectively, strongly suggests that CRP is a programmer for immunosuppressive phenotypes during myeloid development. Furthermore, the requirement for the progenitors to be in S phase suggests that CRP receptor-mediated signals to the progenitors during their most functionally active time. As a consequence, the CRP-regulatory-program is greatest during emergency or demand-adapted myelopoiesis.

We have some evidence of this mechanism participating *in vivo* at baseline and following acute phase induction of CRP. First, we observed that the CRP knockout mouse ($CRP^{-/-}$) has increased blood circulating neutrophils compared to WT and CRPtg mice at baseline (Figure 3). Furthermore, this seems to be a conserved function of CRP as the blood neutrophil frequencies appear similar between WT and CRPtg (mice with mouse CRP and human CRP, respectively, expressed at similar levels[46, 157-159]. Unexplored in $CRP^{-/-}$ mice is whether this is a selective increase in neutrophils or there is a pan-increase in myeloid production. Whether this increase in neutrophils may be

involved in the high resistance of $CRP^{-/-}$ to our AKI model [153] remains to be determined. Additionally, functional differences of the neutrophils (and other myeloid cells) between the CRP mutants is needed. However, these data reveal that CRP dampens neutrophil production at baseline when CRP expression is at lower levels.

AKI in CRPtg induces acute phase levels of CRP and a concomitant diminution of DC populations in the injured kidney compared to WT (Figure 3). Furthermore, there is an increase in $CD11b^{+}Gr\text{-}1^{+}$ populations, which we now know to be constituted by MDSCs[153]. These few *in vivo* observations reinforce the *in vitro* culture data: CRP can potently modulate myeloid development and does so with real biological outcomes, e.g. regulation of neutrophil production and exacerbation of AKI through the over-production of $CD11b^{+}Gr\text{-}1^{+}$ MDSCs.

Myeloid progenitors are known to express FcRs, including FcγRs, at different stages of differentiation [147, 160, 161] and CRP inhibited monocyte precursors that express FcRs [154]. Similarly, I investigated whether CRP needed FcγRIIB to promote MDSCs in BM cultures. Interestingly, CRP did not need FcγRIIB to promote the generation of MDSCs and decrease the preponderance of DCs. There are a couple conjectures that can consolidate these findings. First, CRP programs myelopoiesis through some other FcR (e.g. FcγRI, FcγRIII, or FcαRI) or another receptor altogether. Second, progenitor commitment can be broken down into more refined steps and it is possible that CRP has a greater effect on these precursors (i.e. those not depicted in Figure 2). Identification of receptor involvement in CRP's directorial role in myelopoiesis would allow for specific targeting and mediation of the cellular composition needed for disease resolution.

To date, these reports have focused on monocyte/macrophages, DCs, neutrophils, and MDSCs. Exploration on CRP's effect on other hematopoietic development is needed. Especially in light of the $CRP^{-/-}$ data (Figure 3) it is worth investigating the effect of CRP on the generation of other granulocytes, eosinophils and basophils. There are a few reports of CRP modulating other cell type differentiation on endothelial cell progenitors[162] and that CRP can inhibit B cell colony formation[113]. These studies and our own they clearly demonstrate the need for further research as they lack the rigorous lineage tracking to definitively quantitate the effect of CRP as an innate mediator that dampens the ability immunity to stimulate an adaptive response.

To my knowledge, this is the first study to link CRP as a programmer of myeloid production within a heterogeneous, unsynchronized pool of BM progenitors. Observations from both *in vivo* and *in vitro* using physiologically relevant concentrations of CRP showed this CRP mechanism is operant during health and disease. With the high likelihood of progenitor-receptor expression involvement, their expression patterns within a disease context could bias the CRP diversion of myelopoiesis or vice versa. This is likely exaggerated over time as CRP levels rise with age[163] and there is less FcR-mediated phagocytosis by monocytes and neutrophils[164]. It may be possible that CRP is a mediator of trained immunity (innate "memory") that over-tolerizes myelopoiesis[165] as CRP serum concentration rises with age. Therefore, using the CRP mutant mice (CRPtg and $CRP^{-/-}$) will serve as useful tools to study the ability to generate regulatory myeloid cells during in health and within disease.

CRP Modulates the Functions of Differentiated Myeloid Cells

I propose that the effects of CRP on myelopoiesis and its effects on differentiated, mature myeloid cells are two separate mechanisms. These different mechanisms add to the difficulty in interpreting the flavor of CRP's function as either pro-inflammatory or anti-inflammatory. The context matters and likely comes down to the receptor portfolio, particularly with regard to FcγRs[166], on the cell CRP is acting upon. The receptors' expression level, affinity for CRP, and signaling cascades are all competing and dictate the resultant cellular response to CRP. In the following section I will discuss first the effect of CRP on DCs, MDSCs, macrophages, and neutrophils and second the receptor involvement in these CRP-mediated effects.

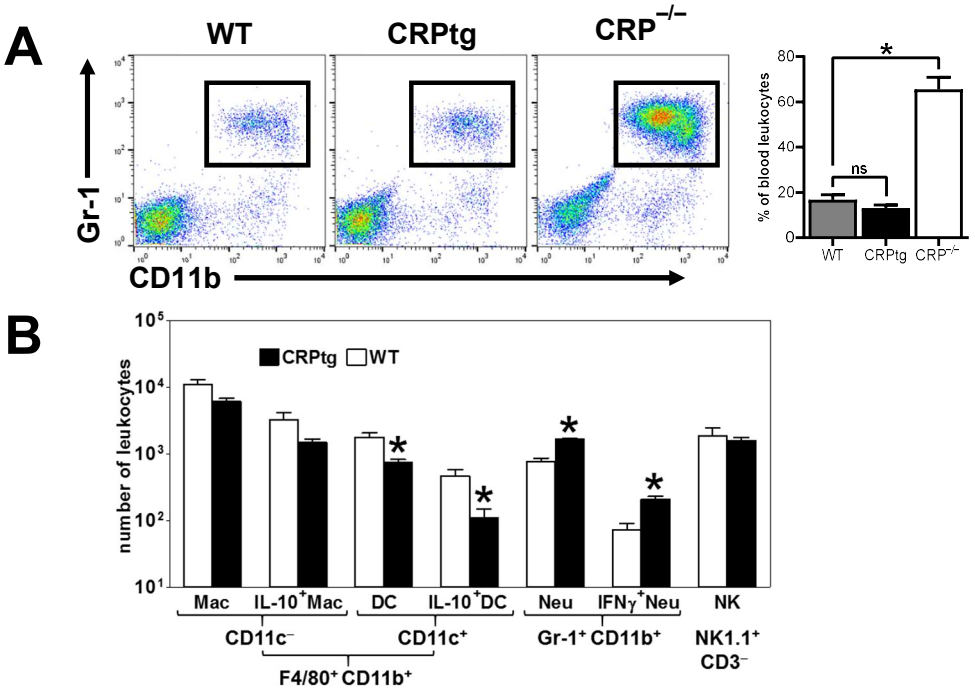


**Figure 3. CRP modulates myeloid development *in vivo* at baseline and post-acute phase response.** (A) CRP knockouts have a high frequency of circulating neutrophils ($CD11b^{+}Gr\text{-}1^{+}$). Representative plots of blood leukocytes from WT, CRPtg, or $CRP^{-/-}$ mice at baseline; mean + SEM of n = 2 mice per genotype compared to WT neutrophils with a one-way ANOVA significance (*) of $p < 0.05$. (B) 24 h following bilateral renal ischemia/reperfusion injury, the kidney infiltrated leukocytes were enumerated from WT (white bars) and CRPtg (black bars). Transcardial perfusion eliminated intravascular cells. Data are the mean + SEM of n = 5 mice and significance (*) $p <$ 0.005 determined by t-tests. Data are courtesy of M. McCrory, M. Pegues, PhD, and A. Szalai, PhD and used with permission.

In separate experiments using MDSCs or DCs grown without CRP, CRP had similar tolerogenic effects. Meaning, CRP-naïve antigen-loaded DCs stimulated T cell proliferation; however, if CRP was added to these co-culture conditions, the DCs no longer stimulated T cell proliferation. Conversely, CRP-naïve MDSCs inhibited the proliferation of CD3/CD28 stimulated T cells; however, if the number of MDSCs added to the co-culture was at an inhibitory-suboptimal ratio (e.g. 1 MDSC: 20 T cells, where T cells still proliferated to some degree), CRP restored the MDSC suppressive function on the CD3/CD28 stimulated T cells. In both *in vitro* experiments with both cell populations, CRP required FcγRIIB expression by the myeloid cell. DCs lacking FcγRIIB were able to stimulate T cell proliferation in the presence of CRP, i.e. they were now CRP refractory. Similarly, CRP could not enhance the suppressive function of FcγRIIB$^{-/-}$ MDSCs. In the former studies, CRP acts as an inhibitor of DC antigen presentation functions and in the latter setting, CRP acts as a promoter of immunosuppressive cellular functions. In both cases, CRP was not present in the maturation/generation of these myeloid lineages, yet CRP had potent and real consequences on the function of these cell types. These two instances highlight how CRP acts as tonic suppressor of immune reactions by i) dampening the stimulatory capacity of DCs and ii) heightening the functions of MDSCs, which results in the indirect inhibition T cell stimulation and direct inhibition of T cell proliferation, respectively. The direct actions of CRP on differentiated, mature myeloid cells ultimately results in a reduced capacity to stimulate or an increased suppression of adaptive immunity.

The discovery of CRP in humans with pneumonia biased the early focus of CRP research. It was first established that CRP is a potent bacterial opsonin for many bacterial

species[167] which lead researchers determine the role of CRP on phagocytosis and their respiratory burst. Indeed, CRP alone enhanced phagocytic functions of neutrophils and monocytes/macrophages and CRP amplified their complement-stimulated phagocytosis[23, 112, 168-171]. Zeller et al. showed that aggregated CRP, and not native CRP, enhanced the respiratory burst of IgG-stimulated monocytes or neutrophils, as assessed by luminol chemiluminescence[172, 173]. Follow-up studies determined that this ability of aggregated CRP to increase aggregated IgG-stimulated reactive oxygen production by monocytes and neutrophils is through the selective induction of intracellular peroxide that is not released into the extracellular space, is not due to any scavenging ability of aggregated CRP, and is mediated through FcγRIIB[170, 174]. Similarly, I showed that CRP does not induce a respiratory burst by either mouse BM derived MDSCs or primary blood circulating neutrophils. However, I showed that intracellular ROS production by mouse MDSCs can be stimulated with CRP that is not heat-aggregated[152]. It is unclear what underlies these differences in observations between aggregated CRP and not-aggregated CRP; it is likely that in the experiments by Zeller et al. the aggregated CRP is engaging multivalent binding to receptors that resulted in a greater and detectable signal and the native CRP in their experiments likely generated a biological response that was below the threshold of detection. However, both their laboratory and ours show that CRP is a selective modulator of ROS biogenesis but the exact mechanism remains to be defined.

To my knowledge, we are the first to show that CRP bestows immunosuppressive capacity to differentiated primary human neutrophils, but only significantly at 100 μg/ml. We also show that CRP increased their intracellular ROS production, and did so faster and more potently at 100 μg/ml. Such a high concentration of CRP would only be present

during acute inflammatory settings and may represent another mechanism by which CRP suppresses the immune response in neutrophilic reactions.

Other studies showed that after 24 h stimulation with 25 μg/ml CRP primary circulating monocytes had increased cell cycle arrest and apoptosis[175]. In light of the data discussed above, it is possible that CRP may be increasing monocyte phagocytosis and/or respiratory burst which, as a consequence, results in their demise. In this case, CRP is not uniquely potentiating cell death, rather it is accelerating their functional demise. As such, the interpretation of CRP's role as an inflammatory mediator depends on the context for the host response: does the immune response need pattern recognition, potent effector cell functions, or a decrease in adaptive response mediated by innate cell types?

## CRP Inhibits Autoimmunity

The *in vivo* consequences of CRP modulation of myeloid cell development and their functions is inferred from its role in autoimmunity. Our laboratory and others have shown that CRP inhibits the development of systemic lupus erythematosus (SLE), rheumatoid arthritics (RA), and multiple sclerosis (MS). Below is a brief overview of the protective effects of CRP in autoimmune models using the stalwart CRPtg mouse.

### *CRP Protects from Lupus Pathogenesis*

As extensively stated, CRP is clinically used as a biomarker of inflammation. However, despite inflammatory processes involved in SLE pathogenesis, SLE patients' CRP levels do not increase or remain low[176-178] yet CRP is known to bind to the U1 small nuclear ribonucleoprotein[31, 179] and contains a nuclear localization motif (Figure 7)[180]. With SLE largely attributed to nuclear antigen antibody development, aggregation, and

deposition, the role of CRP in mediating this pathogenesis was investigated. Indeed, despite the low serum levels of CRP in SLE, CRP was found in immune complexes purified from SLE patients. Furthermore, in lupus prone NZB/NZW mice the administration of human CRP aided in the clearance of nucleosome core-particles and chromatin but showed a short-term protective effect[181, 182]. To investigate this protective role of CRP more robustly, the NZB/NZW mice were crossed with CRPtg mice. These crossed mice showed reduced proteinuria, lived longer, had lower autoantibody titers, had delayed nephropathy, and the pathology did not progress into the renal cortex[183]. These data suggest that the protective effect of CRP is by hiding the autoantigens from B cells and preventing their auto-reactively. CRP could be achieving this by binding to inflamed/ apoptotic tissue expressing phosphocholine and their nuclear constituents. Further in support of this, Marjon et al. investigated the protective effect of CRP in anti-CD41 induced thrombocytopenia, an immune complex-mediated disease, and showed that administration of CRP-treated splenic or bone-marrow derived macrophages treated were protective[184]. Additionally, FcγRI expression on the macrophages was needed for CRP to program protective macrophages. These data suggest that in SLE CRP is directly promoting FcγRI$^{+}$ macrophage phagocytosis of circulating immune complexes and is also mediating protection through ligand binding.

*CRP Protects from Arthritis Development*

In rheumatoid arthritis (RA) patients, their serum CRP level is found within the arthritic joint[96, 185] and synovial fluid[186], correlates with disease and progression[187], and thus is incorporated into clinical computation of RA disease activity[188]. These clinical data strongly suggest CRP is detrimental in RA. Contrastingly, using a collagen-induced

arthritis model (CIA), Jones et al. showed that CRPtg mice were protected from CIA, $CRP^{-/-}$ mice had accelerated and more severe CIA, yet both mice showed equal amounts of anti-collagen antibodies[127]. These data show that during onset and development of disease, CRP plays a protective role. Interestingly, direct immunization with arthritogenic antibodies into $CRP^{-/-}$ and WT mice had no difference in disease, suggesting that CRP is dampening the initial immunogenicity of adaptive immunity. The human data suggest that following establishment of disease and pathology, CRP actions are deleterious. A conjecture for CRP involvement in RA is that, in contrast to SLE, CRP protection is not through ligand binding of autoantigens, but rather is promoting phagocytosis of inflamed tissue and decreasing DC activation thereby lowering T cell activation and their inflammatory cytokine production.

*CRP Protects from Multiple Sclerosis*

The CRP protective effect in autoimmunity models of lupus, arthritis, and thrombocytopenia suggest a universal role for CRP as a tonic suppressor of autoimmunity. This hypothesis was further testing in the multiple sclerosis model, experimental autoimmune encephalomyelitis (EAE). Indeed, CRPtg mice had delayed and less severe EAE induced either by active immunization with immunodominant myelin oligodendrocyte (MOG) peptide ($MOG_{33-55}$) or by passive induction through administration of encephalitogenic T cells from WT mice with active EAE[189]. Additional studies showed that CRP protection was mediated by FcγRIIB on $CD11c^+$ cells (likely DCs)[123, 125]. These data suggest that part of CRP's protection is by signaling through FcγRIIB on $CD11c^+$ DCs to reduce their activation and maturation, thereby preventing their ability to present antigen and promote the expansion of autoreactive T cells.

Unexplored is the hypothesis proposed in the preceding section: does CRP redirect the myelopoiesis in autoimmunity; does the CRP effect change with initiation, establishment, and progression of disease? This could in part aid in clarifying clinical associations with CRP, as with RA wherein CRP is first delays pathology but after established disease likely promotes autoimmunity.

CRP inhibition of DC APC functions is likely participant during health and strongly suggests that CRP is a tonic suppressor of aberrant adaptive immunity, i.e. autoimmunity. Furthermore, its prevention of lupus is greater due to its ability to bind inflamed tissue and the nuclear autoantigens implicated in lupus etiology. In these contexts, CRP can be classified as anti-inflammatory because this suits the health outcome we humans desire: reduction of misdirected immunogenicity. However, in disease settings wherein the generation of regulatory immune response is not desired, CRP is pro-inflammatory, such as AKI.

## CRP Exacerbates AKI

Acute kidney injury (AKI) induced by renal ischemia reperfusion can occur through unknown origins, occurs with myocardial infarction[190], and is inherent in the renal transplant setting[190, 191]. Although not clearly defined within this injury context, inflammation is participant. Not surprisingly, high serum CRP i) positively correlates with decreased renal function[192, 193] and increased renal injury markers post-AKI[194, 195], ii) are increased in chronic kidney disease (CKD) patients 196, iii) when high in kidney transplant recipients predicts their graft failure[197, 198], and iv) predicts overall mortality [199, 200]. This wealth of association suggested that CRP actions in AKI are detrimental; these

clinical associations are similar to those observed in RA patients however, animal studies showed that CRP deleterious actions are dependent upon the timing of the disease.

To clarify the role of CRP in AKI our laboratory surgically induced bilateral renal ischemia/reperfusion injury, a preclinical model of AKI, in our CRPtg and $CRP^{-/-}$ mice. The CRPtg clearly had worse kidney damage compared to WT and $CRP^{-/-}$ mice: they had increased cast formation, tubular necrosis, epithelial brush border loss, serum creatinine, urine albumin[109, 153]. This exacerbation of AKI was prevented with prophylactic administration of a CRP antisense oligonucleotide (CRP ASO). Coincident with this CRP ASO treatment was a reduction in kidney infiltrating $CD11b^{+}Gr\text{-}1^{+}$ cells and contrastingly, CRPtg had increased $CD11b^{+}Gr\text{-}1^{+}$ cells in their kidneys compared to WT and $CRP^{-/-}$. We ascertained that these cells include myeloid derived cells with suppressor phenotypes (MDSC) as when these $CD11b^{+}Gr\text{-}1^{+}$ kidney infiltrating cells were sorted and used in co-cultures, they inhibited the proliferation of CD3/CD28 stimulated T cells[153]. Furthermore, we showed that their frequency decrease in the bone marrow post-AKI with no change in spleen frequency. Therefore, we theorized that CRP likely effects in AKI by i) directing myeloid development, ii) altering the function of the MDSCs through FcγR(s), and iii) regulating the immune response through ligand binding, e.g. PC on dying cells.

The majority of my dissertation work sought to connect the dots, to investigate whether CRP directly modulates MDSC development and function and in turn whether the MDSCs have a noxious effect on a renal cell type. As discussed above, I showed that CRP increases the generation of $CD11b^{+}Gr\text{-}1^{+}$ cells (MDSCs) from BM progenitors. Taken *in vivo*, these data suggest that CRP increases the generation of MDSCs from the BM which then infiltrate the injured kidney. Furthermore, CRP increased MDSC

intracellular ROS and also increased their ability to inhibit CD3/CD28 stimulated T cell proliferation, suggesting that CRP enhances a ROS-mediated suppression mechanism employed by MDSCs.

After establishing the direct effect of CRP on MDSCs, the next series of experiments (reported in the chapter, *Myeloid derived cells with suppressor functions impair renal tubular epithelial cell cycling*) explored whether MDSCs effect primary renal tubular epithelial cells (RTEC). I chose to generate and study RTECs since during renal ischemia/reperfusion the early proximal tubular epithelial cells are prone to the hypoxic damage, suggesting a possible link between the MDSC-produced ROS and CRP heightening these effects. In this study, I show that the ability of RTECs to progress through S phase of the cell cycle was impaired by MDSCs both with and without cell contact. The consequence of RTEC stalling does not result in their cell death as they showed no increase in apoptosis nor necrosis (data not shown) following co-culture with MDSCs. Proper renal epithelial proliferation is key to ensure proper healing and decrease the likelihood of maladaptive repair (i.e. fibrosis). As we showed previously, CRPtg mice have increased tubular necrosis and brush border loss, indicating that the renal epithelium is particularly injured. Therefore, it is probable that CRP i) increases the generation of MDSCs, ii) as a consequence, more MDSCs are able to infiltrate the injured kidney as compared to WT, and iii) once in the kidney, MDSCs inhibit the ability of the renal epithelia to reseed the damaged tubules. Ultimately, not only is there worse initial injury but also increased fibrosis and decreased renal filtration function that progresses to chronic kidney disease. Indeed, in a chronic kidney disease model, unilateral ureteral obstructive nephropathy, CRPtg mice had more fibrosis development[201].

The MDSC soluble mediator is unknown and CRP does not enhance its effects on RTEC S phase inhibition. It is unlikely to be ROS or RNS and may be cytokine mediated. Future studies will need to determine more widely what the MDSCs are secreting to impair RTECs. Targeting this soluble mediator could prevent the detrimental actions of the MDSCs on RTEC cell cycling and may alleviate the chance that fibrosis occurs. Lowering CRP expression within 24 h of AKI or within the kidney graft recipient would lower MDSC generation, infiltration, and contribution to the hypoxic damage.

The results presented within this section may appear counterintuitive: CRP is promoting the generation and actions of a pro-inflammatory myeloid population that aggravates AKI. However, from the standpoint of CRP it may be erroneously producing a regulatory myeloid population in attempts to quell the inflammation stimulated by the severe injury. Additionally, the simple statement that there is "inflammation" due to high myeloid cell content does not fully describe the nuances to the cellular composition. For example, macrophage efferocytosis of apoptotic and necrotic debris is known to aid in the resolution of injury[202]. It is likely that there is a balance of activating and regulatory myeloid cells that are needed at every stage of the inflammatory response following AKI. Unfortunately, CRP tips the scales towards regulatory myeloid cells (MDSC). While I show evidence of the effects MDSCs have on renal epithelia it is acknowledge that there is wide variety of cell types involved in AKI. It is possible that MDSCs have inhibitory effects on renal stromal cells and on both renal-resident and infiltrating leukocytes which aim to resolve and repair the damage. Indeed, MDSCs are known to injury podocytes[203] and modulate cytokine production by macrophages[204]. This non-specific suppression by MDSCs is widely studied and described for tumor associated MDSCs.

## CRP Associates with Poor Cancer Prognosis

High CRP levels associate with increased risk[205-207], progression[208], and mortality [209, 210]. In many analyses, CRP is an independent prognostic factor[211-216] and highlight the importance of investigating the biological functions of CRP during cancer[64, 217].

Despite the success of solid tumor treatment with immunotherapy, e.g. checkpoint inhibitors and chimeric antigen receptor (CAR) T cells, there is a significant fraction of patients that experience no effect or experience a short remission. Immunotherapy research appears to have a two pronged approach, i) boosting T cell anti-tumor responses to overcome antigen escape and T cell exhaustion following immunotherapy[218] and ii) modulate intra-tumoral MDSCs as a synergistic treatment[219]. MDSCs were first described in the cancer setting because of their ability to suppress T cells and their anti-tumor responses. Therefore, reprogramming the function of MDSCs could aid in T cell-driven neoplasm clearance and improve the efficacy of current immunotherapies. Our *in vitro* and *in vivo* data show CRP as a modulator of MDSC generation and suppressive function and thus targeting CRP expression is promising to achieve such a goal.

In a joint pilot study between the Szalai and Norian laboratories we challenged the CRP mutant mice with an orthotopic breast cancer model (E0771). We measured tumor area and enumerated leukocyte populations in the tumors and spleens in WT, $CRP^{-/-}$ and CRPtg mice. Based on the thesis presented in the beginning of this dissertation discussion we expected that compared to WT the CRPtg would have more MDSCs and more tumor burden with the inverse in $CRP^{-/-}$ mice. While we saw no difference in CRPtg versus WT, we saw that $CRP^{-/-}$ followed our prediction: they had lower tumor outgrowth and lower frequencies of MDSCs in the tumor and spleen. These promising data demonstrate the possibility that CRP contributes to MDSC generation and

their eventual infiltration into the tumor. Unexplored is whether in the tumor micro-environment CRP impedes anti-tumor responses directly (i.e. inhibiting T cells per se) or indirectly (i.e. promoting MDSC immunosuppression). The translational ability of these studies can be easily done with the proven and efficacious CRP antisense oligonucleotide that works equally in CRPtg mice and in humans[59].

In conclusion, I posit that CRP, an evolutionarily conserved pattern recognition molecule, acts as baseline and potently during the APR, to program a regulatory phenotype during the developmental stages of myeloid production. This cellular arm of CRP's suppression of immunity remains to be elaborated. Myeloid derived cells with suppressor phenotypes (MDSC) may likewise be evolutionarily conserved as a mechanism to dampen severe and chronic immune reactions. Both CRP and MDSCs are oft mischaracterized as bad players. I argue that this view is too simplistic and is dependent upon the context. In autoimmunity, both CRP and MDSCs likely aid in tolerizing the immune response to self and aim to turn off the misguided immunity. In acute injury or trauma, the beneficial role of CRP and MDSCs would increase with the gradation of severity however their benefit likely reaches a threshold, at which point they would instead exacerbate the pathology. In chronic diseases such as cancer, CRP and MDSCs appear to have a detrimental role by allowing the tumor to persist yet trying to allay the constant immune activation. Should this thesis hold true, CRP represents a highly druggable target to reset the balance of myeloid development, provides a cellular context to its many clinical associations, and becomes more than a mere biomarker of inflammation.

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# APPENDIX A

# SUPPLEMENTARY MATERIAL FOR: INTRODUCTION

**Table A. CRP protein ortholog pairwise identity compared to human CRP**

| | | |
|---|---|---|
| 1 | NP_000558.2 | *Homo sapiens* |
| 2 | XP_001170732.2 | *Pan troglodytes* |
| 3 | XP_003821015.1 | *Pan paniscus* |
| 4 | XP_004027118.1 | *Gorilla gorilla gorilla* |
| 5 | XP_002809982.1 | *Pongo abelii* |
| 6 | XP_003258733.1 | *Nomascus leucogenys* |
| 7 | XP_010359092.1 | *Rhinopithecus roxellana* |
| 8 | XP_011812576.1 | *Colobus angolensis palliatus* |
| 9 | XP_023070170.1 | *Piliocolobus tephrosceles* |
| 10 | XP_017720378.1 | *Rhinopithecus bieti* |
| 11 | XP_007974764.1 | *Chlorocebus sabaeus* |
| 12 | XP_011825813.1 | *Mandrillus leucophaeus* |
| 13 | XP_011768322.1 | *Macaca nemestrina* |
| 14 | XP_009182747.1 | *Papio anubis* |
| 15 | NP_001306322.1 | *Macaca fascicularis* |
| 16 | XP_001117250.2 | *Macaca mulatta* |
| 17 | XP_011923089.1 | *Cercocebus atys* |
| 18 | XP_025244391.1 | *Theropithecus gelada* |
| 19 | XP_012305138.1 | *Aotus nancymaae* |
| 20 | XP_002760205.2 | *Callithrix jacchus* |
| 21 | XP_003937959.1 | *Saimiri boliviensis boliviensis* |
| 22 | XP_017359161.1 | *Cebus capucinus imitator* |
| 23 | XP_012517316.1 | *Propithecus coquereli* |
| 24 | XP_008056920.1 | *Carlito syrichta* |
| 25 | XP_008567757.1 | *Galeopterus variegatus* |
| 26 | XP_012607845.1 | *Microcebus murinus* |
| 27 | XP_003795254.1 | *Otolemur garnettii* |
| 28 | XP_012886039.1 | *Dipodomys ordii* |
| 29 | XP_015347516.1 | *Marmota marmota marmota* |
| 30 | XP_003355155.1 | *Sus scrofa* |
| 31 | NP_001075734.1 | *Oryctolagus cuniculus* |
| 32 | XP_005339443.1 | *Ictidomys tridecemlineatus* |
| 33 | XP_027806500.1 | *Marmota flaviventris* |
| 34 | XP_003466601.1 | *Cavia porcellus* |
| 35 | XP_026263752.1 | *Urocitellus parryii* |
| 36 | XP_004858808.1 | *Heterocephalus glaber* |
| 37 | XP_006061078.1 | *Bubalus bubalis* |
| 38 | XP_028641555.1 | *Grammomys surdaster* |
| 39 | XP_031198415.1 | *Mastomys coucha* |
| 40 | XP_006990626.1 | *Peromyscus maniculatus bairdii* |
| 41 | XP_021055034.1 | *Mus pahari* |
| 42 | XP_010614994.1 | *Fukomys damarensis* |
| 43 | XP_003500282.1 | *Cricetulus griseus* |
| 44 | XP_010845159.1 | *Bison bison bison* |
| 45 | XP_027384786.1 | *Bos indicus x Bos taurus* |
| 46 | XP_019843940.1 | *Bos indicus* |
| 47 | NP_001137569.1 | *Bos taurus* |
| 48 | XP_004688136.1 | *Condylura cristata* |
| 49 | NP_031794.3 | *Mus musculus* |
| 50 | XP_021018969.1 | *Mus caroli* |
| 51 | XP_017901842.1 | *Capra hircus* |
| 52 | XP_028721465.1 | *Peromyscus leucopus* |
| 53 | XP_005078251.1 | *Mesocricetus auratus* |
| 54 | XP_008839057.1 | *Nannospalax galili* |
| 55 | XP_025770918.1 | *Puma concolor* |
| 56 | XP_004442822.1 | *Ceratotherium simum simum* |
| 57 | XP_014939271.2 | *Acinonyx jubatus* |
| 58 | XP_015104515.1 | *Vicugna pacos* |
| 59 | XP_006895510.1 | *Elephantulus edwardii* |
| 60 | XP_007084342.1 | *Panthera tigris altaica* |
| 61 | XP_026343136.1 | *Ursus arctos horribilis* |
| 62 | XP_008518716.1 | *Equus przewalskii* |
| 63 | XP_008698934.1 | *Ursus maritimus* |
| 64 | XP_014703803.1 | *Equus asinus* |
| 65 | XP_003999711.3 | *Felis catus* |
| 66 | XP_001504452.1 | *Equus caballus* |
| 67 | XP_002929488.1 | *Ailuropoda melanoleuca* |
| 68 | XP_030158335.1 | *Lynx canadensis* |
| 69 | XP_019288899.1 | *Panthera pardus* |
| 70 | XP_004671218.1 | *Jaculus jaculus* |
| 71 | XP_021537599.1 | *Neomonachus schauinslandi* |
| 72 | XP_005368614.1 | *Microtus ochrogaster* |
| 73 | NP_058792.1 | *Rattus norvegicus* |
| 74 | XP_004407907.1 | *Odobenus rosmarus divergens* |
| 75 | XP_006742024.1 | *Leptonychotes weddellii* |
| 76 | XP_017500302.1 | *Manis javanica* |
| 77 | XP_029082678.1 | *Monodon monoceros* |
| 78 | XP_004589327.1 | *Ochotona princeps* |
| 79 | XP_025716525.1 | *Callorhinus ursinus* |
| 80 | XP_019788299.1 | *Tursiops truncatus* |
| 81 | XP_006168911.1 | *Tupaia chinensis* |
| 82 | XP_012393934.1 | *Orcinus orca* |
| 83 | XP_016070828.1 | *Miniopterus natalensis* |
| 84 | XP_029789060.1 | *Suricata suricatta* |
| 85 | XP_025842079.1 | *Vulpes vulpes* |
| 86 | XP_026950737.1 | *Lagenorhynchus obliquidens* |
| 87 | XP_025276447.1 | *Canis lupus dingo* |
| 88 | XP_003767916.2 | *Sarcophilus harrisii* |
| 89 | NP_001301045.1 | *Canis lupus familiaris* |
| 90 | XP_018415276.1 | *Nanorana parkeri* |
| 91 | XP_005442646.1 | *Falco cherrug* |
| 92 | XP_005244420.1 | *Falco peregrinus* |
| 93 | XP_009567099.1 | *Cuculus canorus* |
| 94 | XP_027821246.1 | *Ovis aries* |
| 95 | AGD81192.1 | *Cynoglossus semilaevis* |
| 96 | CCO02601.1 | *Oncorhynchus mykiss* |
| 97 | AKR17056.1 | *Sebastes schlegelii* |
| 98 | NP_001134140.1 | *Salmo salar* |
| 99 | ACI66342.1 | *Salmo salar* |
| 100 | AET80950.1 | *Danio rerio* |
| 101 | XP_017332852.1 | *Ictalurus punctatus* |
| 102 | AEU04519.1 | *Cyprinus carpio carpio* |
| 103 | AKO22072.1 | *Carassius auratus* |
| 104 | AAA28270.1 | *Limulus polyphemus* |

**Table B. The number of helix turns for the CRP intron polymorphic $(GT)^n$ repeats and flanking sequence.**

| | number of helix turns | |
|---|---|---|
| $n^a$ | $(GT)^{n\ b}$ | +flanking |
| 9 | 1.5 | 5.9 |
| 10 | 1.7 | 6.1 |
| 11 | 1.8 | 6.2 |
| 12 | **2** | 6.4 |
| 13 | 2.2 | 6.6 |
| 14 | 2.3 | 6.7 |
| 15 | 2.5 | 6.9 |
| 16 | 2.7 | 7.1 |
| 17 | 2.8 | 7.2 |
| 18 | **3** | 7.4 |
| 19 | 3.2 | 7.6 |
| 20 | 3.3 | 7.7 |
| 21 | 3.5 | 7.9 |
| 22 | 3.7 | 8.1 |
| 23 | 3.8 | 8.2 |
| 24 | **4** | 8.4 |
| 25 | 4.2 | 8.6 |

GT repeat ($(GT)^n$) alleles associated with low (blue) or high (red) serum CRP levels; complete revolutions of the $(GT)^n$ are in bold.

a The number of GT repeats

b Calculated based on the number of bases in the GT repeat in a Z-DNA conformation: 12 base pairs per helix turn

c Additional turns for the flanking 44 bases in B-DNA conformation: 10 bases per helix turns.

Human and mouse CRP amino acid pair-wise alignment

```
Percentage ID = 69.78
Score = 8610.0
Human 224 aa
Mouse 225 aa

HUMAN MEKLL-CFLVLTSLSHAFGQTDMSRKAFVFPKESDTSYVSLKAPLTKPLKAFTV
      ||||| |.|.. |.|..||. || .|||||||||||||||| |   |||..|||
MOUSE MEKLLWCLLIMISFSRTFGHEDMFKKAFVFPKESDTSYVSLEAESKKPLNTFTV

HUMAN CLHFYTELSSTRGYSIFSYATKRQDNEILIFWSKDIGYSFTVGGSEILFEVPEV
       |||||| ||. |..|.|||||.. |.|||||.|| |.| |||.|. | |.|.
MOUSE CLHFYTALSTVRSFSVFSYATKKNSNDILIFWNKDKQYTFGVGGAEVRFMVSEI

HUMAN TVAPVHICTSWESASGIVEFWVDGKPRVRKSLKKGYTVGAEASIILGQEQDSFG
        || |||.|||||.||||||.||||.||||| ||||||..||||||||||.|
MOUSE PEAPTHICASWESATGIVEFWIDGKPKVRKSLHKGYTVGPDASIILGQEQDSYG

HUMAN GNFEGSQSLVGDIGNVNMWDFVLSPDEINTIYLGGPFSPNVLNWRALKYEVQGE
       |.|.. ||||||||.||||||||||..|.|.|.|.|| .|||||||||.| ||.
MOUSE GDFDAKQSLVGDIGDVNMWDFVLSPEQISTVYVGGTLSPNVLNWRALNYKAQGD

HUMAN VFTKPQLWP
      || |||||.
MOUSE VFIKPQLWS
```

# APPENDIX B

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

**MEMORANDUM**

**DATE:** 03-Dec-2019

**TO:** Szalai, Alexander J

**FROM:** Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

**SUBJECT: NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 03-Dec-2019.

| | |
|---|---|
| **Protocol PI:** | Szalai, Alexander J |
| **Title:** | Core Center for Acute Kidney Injury Research (Agarwal); C-Reactive Protein in Acute Kidney Injury |
| **Sponsor:** | National Institute of Diabetes and Digestive and Kidney Diseases/NIH/DHHS |
| **Animal Project Number (APN):** | IACUC-10004 |

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 02-Dec-2022.

**Institutional Animal Care and Use Committee (IACUC)**

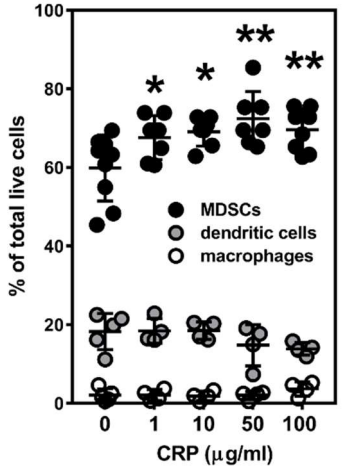
403 Community Health on 19th | 933 19th Street South

Mailing Address:
CH19 403 | 1720 2nd Ave South | Birmingham AL 35294-2041

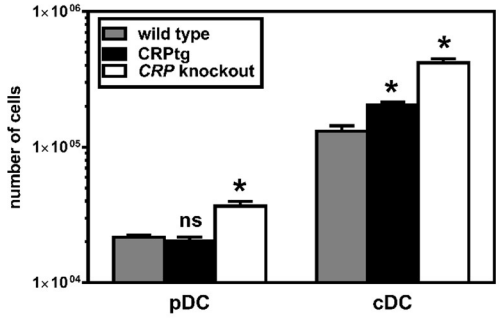
phone: 205.934.7692 | fax: 205.934.1188
www.uab.edu/iacuc | iacuc@uab.edu

# APPENDIX C

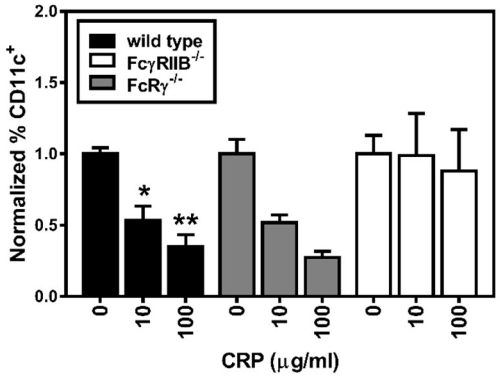
## SUPPLEMENTARY MATERIAL FOR: C-REACTIVE PROTEIN IMPAIRS DENDRITIC CELL DEVELOPMENT, MATURATION, AND FUNCTION: IMPLICATIONS FOR PERIPHERAL TOLERANCE




**Supplemental Figure 1.** Human CRP promotes the generation of bone marrow-myeloid derived suppressor cells (BM-MDSC). Bone marrow cultured under conditions favoring MDSC generation (1) with human CRP added on day 0 and analyzed by flow cytometry. CRP significantly and dose-dependently increased the proportion of MDSCs (●, $CD11c^{-}CD11b^{+}F4/80^{-}Ly6C^{+}Ly6G^{+}$), that appears to be at the expense of dendritic cells (●, $CD11c^{+}CD11b^{+}F4/80^{-}$), with no effect on macrophages (○, $CD11c^{-}CD11b^{+}F4/80^{-}$). One-way ANOVA with multiple comparisons $p < 0.01$ (*) and $p < 0.001$ (**) from n = 7 – 10.




**Supplemental Figure 2.** Loss of CRP expression *in vivo* alters DC subtypes. In the absence of CRP (i.e. in *CRP* knockout mice), the numbers of both pDCs ($CD11c^{+}CD11b^{+/-}$Siglec $H^{+}$) and cDCs ($CD11c^{+}CD11b^{+}$Siglec $H^{-}$) is increased in the spleen. The latter play a major role in $CD4^{+}$ T cell activation. One-way ANOVA with multiple comparisons $p < 0.05$ (*) from n = 3 per genotype.




**Supplemental Figure 3.** CRP impairs the generation of $CD11c^{+}$ BMDCs in an FcγRIIB-dependent manner. BMDCs were generated with bone marrow from wild type (WT), $FcγRIIB^{-/-}$, and $FcRγ^{-/-}$ mice. The latter lack the common gamma chain used for signaling by activating FcγRs (FcγRI, FcγRIII, and FcγRIV). CRP significantly and dose-dependently decreased the proportion of $CD11c^{+}$ BMDCs in WT with a similar trend in $FcRγ^{-/-}$ cultures; this CRP effect is absent in $FcγRIIB^{-/-}$ BMDC cultures. Each genotype is normalized to those cultures that were never exposed to CRP. Two-way ANOVA with Dunnett's multiple comparisons test versus 0 CRP within each genotype $p < 0.05$ (*) and $p < 0.005$ (**) from n = 3 – 9.

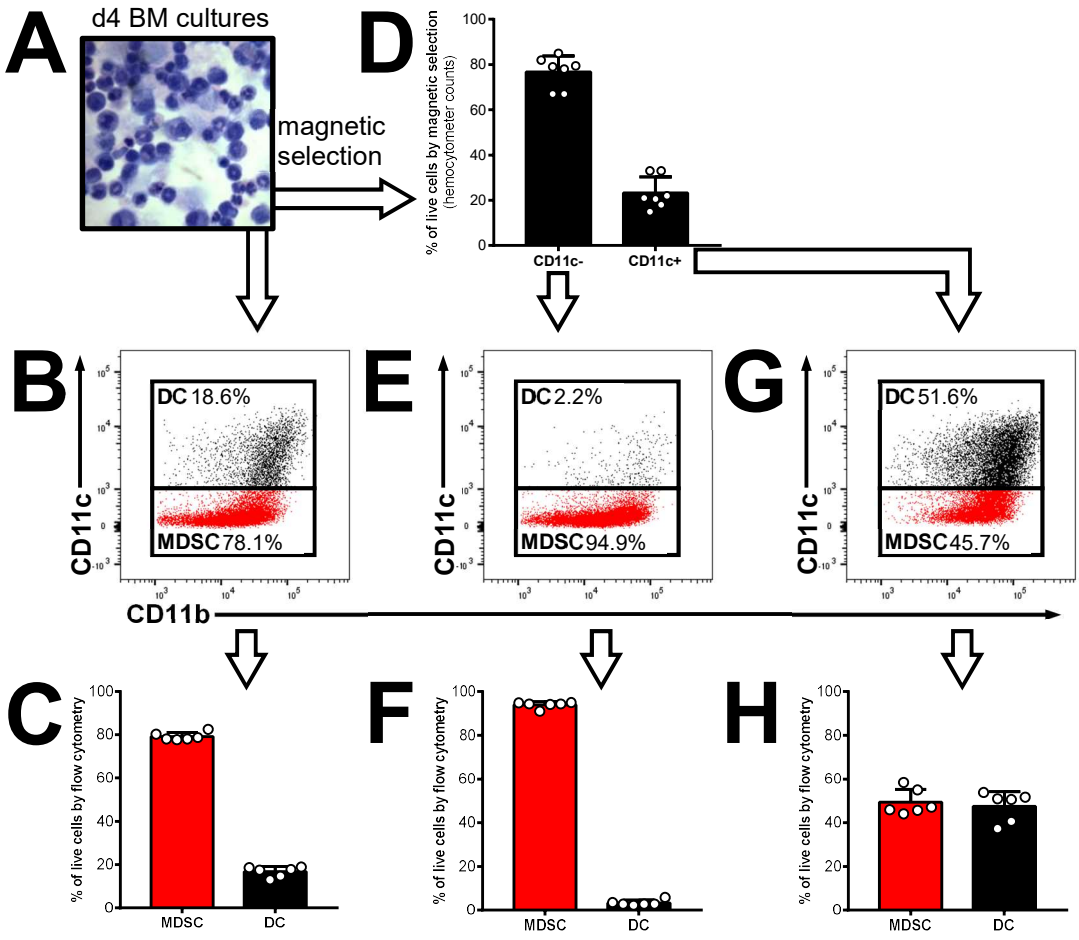
References

1. Höchst, B. et al. Differential induction of Ly6G and Ly6C positive myeloid derived suppressor cells in chronic kidney and liver inflammation and fibrosis. *PloS one* 10, e0119662 (2015).

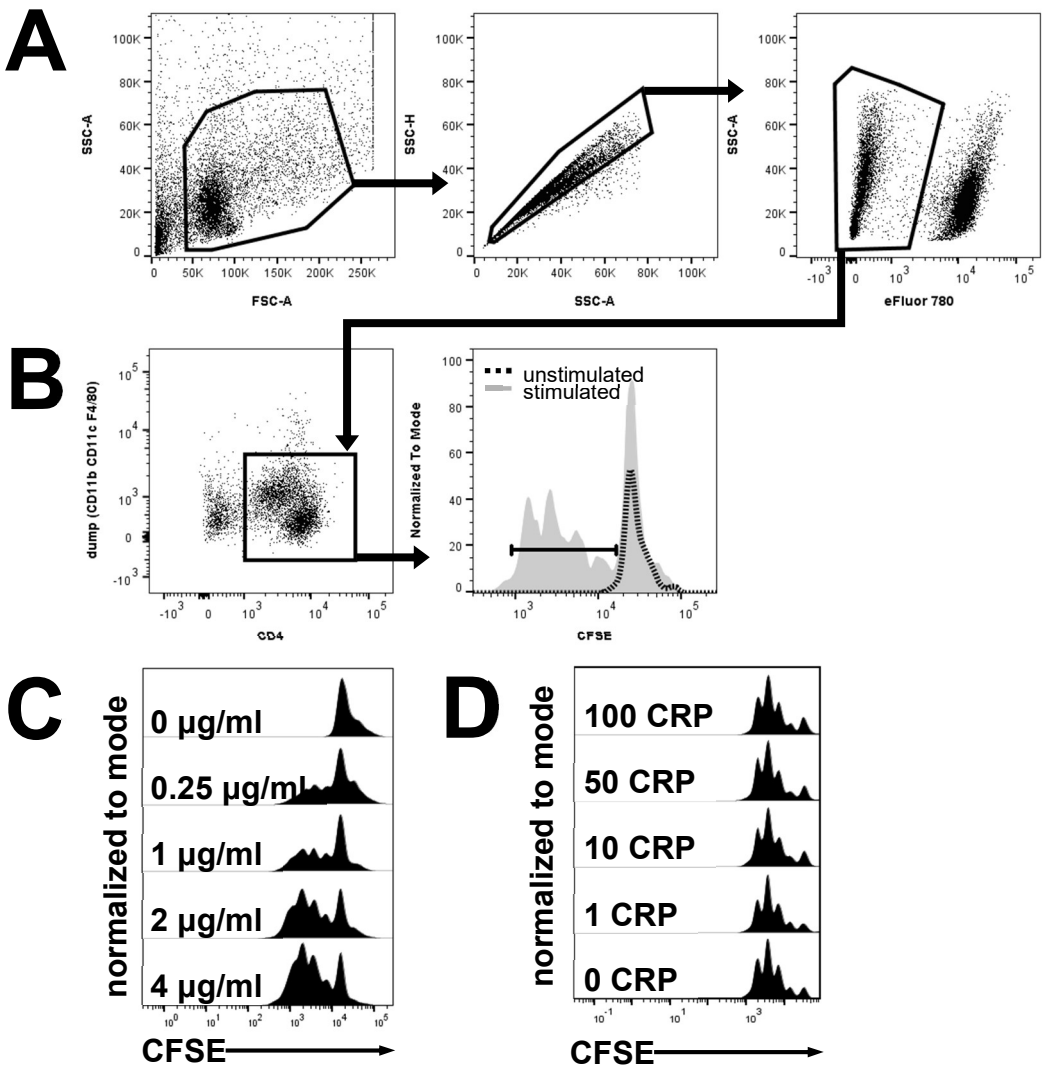
2. Jones, N.R. et al. A Selective Inhibitor of Human C-reactive Protein Translation Is Efficacious In Vitro and in C-reactive Protein Transgenic Mice and Humans. *Mol Ther Nucleic Acids* 1, e52 (2012).

# APPENDIX D

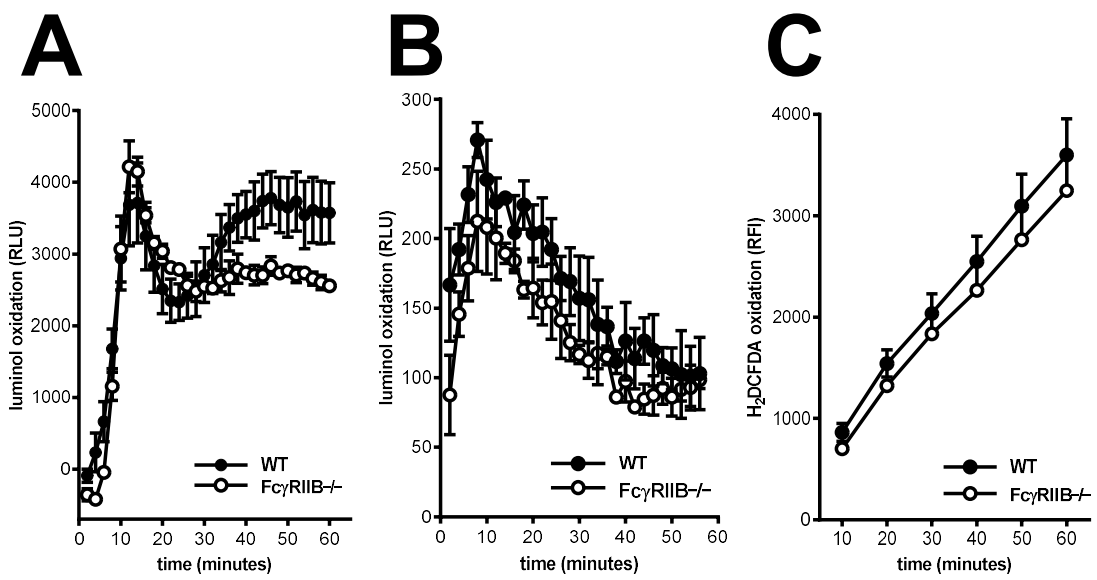
## SUPPLEMENTARY MATERIAL FOR:
## C-REACTIVE PROTEIN PROMOTES THE EXPANSION OF MYELOID DERIVED CELLS WITH SUPPRESSOR FUNCTIONS




**Supplemental Figure 1. Enrichment of MDSCs from mouse bone marrow cultures.** Mouse bone marrow (BM) progenitors were cultured under conditions tailored to generate MDSCs (1). On day 4 of culture the cells (**A**) were subjected to flow cytometry to enumerate $CD11c^{-}$ $CD11b^{+}F4/80^{-}Ly6G^{+}Ly6C^{+}$ MDSCs and $CD11c^{+}CD11b^{+}$ DCs (**B**). MDSCs represented 79.2 ± 1.8% of all live cells in the d4 BM cultures (**C**). Samples from the same cultures were subjected to immunomagnetic selection (see the *Materials and Methods*) and the number of $CD11c^{+}$ cells (DCs; positively selected) and $CD11c^{-}$ cells (MDSCs; negatively selected) were enumerated using a hemocytometer. $CD11c^{-}$ cells represented 76.7 ± 7.1% of all live cells in the d4 BM cultures (n = 7 cultures; panel **D**). Flow cytometry of the negatively selected cells (**E**, **F**) confirmed that the $CD11c^{-}$ $CD11b^{+}F4/80^{-}Ly6G^{+}Ly6C^{+}$ MDSCs were highly enriched (94.0 ± 1.5% of all live negatively selected cells). Note the higher frequency of MDSCs following enrichment (F) compared to the BM-MDSC culture prior to immunomagnetic selection (C). Panels **G** and **H** show that the positively selected fraction is a mixture of DC and MDSCs.




**Supplemental Figure 2. Strategy for identification of proliferating CFSE$^+$CD4$^+$ T cells in BM-MDSC:T cell co-cultures.** Three days after initiating co-cultures of BM-MDSCs plus CD3/CD8 stimulated CFSE-labeled T cells the cells were harvested and processed for flow cytometry. (A) Sequentially (top row of panels from left to right), debris was gated out using an FSC-A × SSC-A plot, doublets were gated out using an SSC-A × SSC-H plot, and eFluor780$^+$ dead cells were gated out. Next using a CD11b$^+$CD11c$^+$ F4/80$^+$ dump gate myeloid cells were excluded (B). The gated CD4$^+$ T cells were then used to generate CFSE histograms (normalized to mode) and unstimulated versus CD3/CD28 stimulated T cells were compared to identify and enumerate proliferating T cells (indicated by the horizontal bracket). Prior to performing T cell proliferation assays each lot of anti-CD3 mAb was titrated and used at concentrations that resulted in 3 to 5 discernable generations of CFSE$^+$CD4$^+$ T cells after 72 h of culture; panel C shows representative CFSE histograms for T cells stimulated with increasing amounts (0 – 4 µg/ml) of anti-CD3 mAb. (D) In preliminary experiments we verified that (in the absence of BM-MDSCs) human CRP (1-100 µg/ml) had no discernable effect on the proliferation of CD3/CD28 stimulated mouse CD4$^+$ T cells.



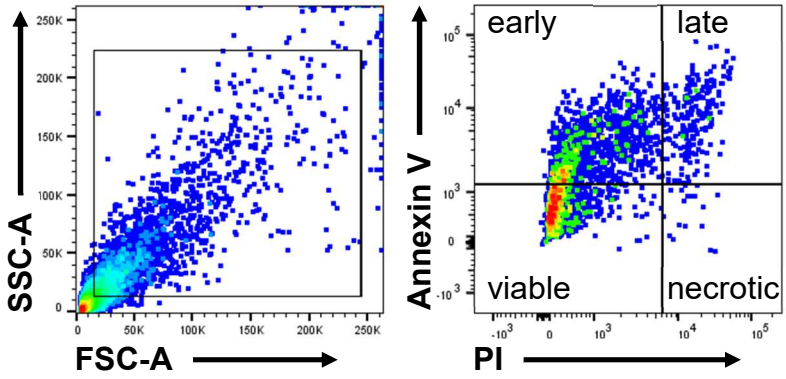

**Supplemental Figure 3.** Loss of FcγRIIB does not significantly alter PMA-induced production of ROS by mouse MDSCs. Mouse MDSCs were generated from bone marrow (BM) supplied by wild type (WT) or FcγRIIB$^{-/-}$ mice to study the receptor's requirement for the generation of ROS. The generation of ROS after stimulation with PMA (100 nM) was not affected by loss of FcγRIIB expression. Extracellular ROS production by (A) BM-MDSCs or (B) enriched MDSCs measured via luminol assay and (C) intracellular ROS production by enriched MDSCs measured via $H_2$DCFDA assay.

Reference

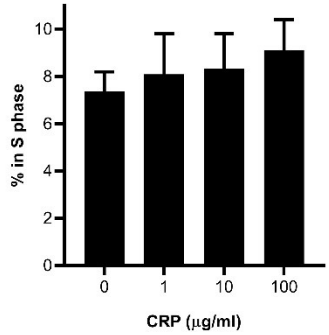
1. Höchst, B. et al. Differential induction of Ly6G and Ly6C positive myeloid derived suppressor cells in chronic kidney and liver inflammation and fibrosis. *PloS one* 10, e0119662 (2015).

# APPENDIX E

## SUPPLEMENTARY MATERIAL FOR MYELOID DERIVED CELLS WITH SUPPRESSOR FUNCTIONS IMPAIR RENAL TUBULAR EPITHELIAL CELL CYCLING




**Supplemental Figure 1. Gating strategy of apoptotic RTECs from transwell co-cultures.** RTECs were harvested and analyzed for Annexin V binding to phosphatidylserine on flipped cell membranes of apoptotic cells.




**Supplemental Figure 2. Human CRP has no effect on RTEC ability to cycle through S phase.** Data are the replicates mean + SEM of separate experiments (n = 11).