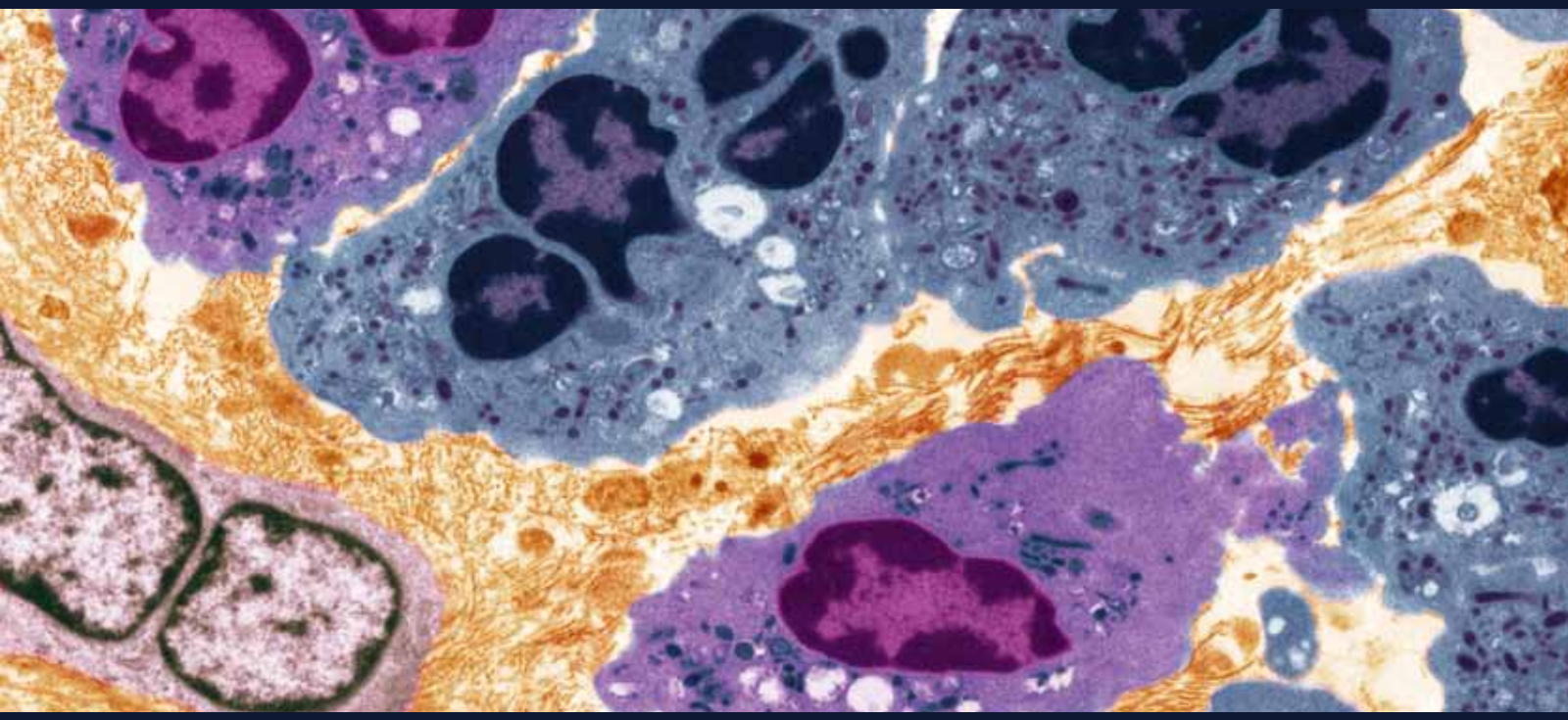
International Journal of Inflammation

Curbing Inflammation

Guest Editors: R. Clive Landis, Christopher D. Buckley, Paulo Roberto B. Evora, and David A. Hart



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Editorial Board

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 468287, 3 pages
http://dx.doi.org/10.1155/2013/468287

Editorial

Curbing Inflammation

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Received 11 July 2013; Accepted 11 July 2013

1. Introduction

Inflammation stands at the centre of a range of natural and pathological processes, such as ageing, wound healing, infection, arthritis, autoimmune disease, cardiovascular disease, cancer, and the inflammatory response to surgery. It is well accepted that inflammation, in the right place and at the right time, is at the center of a healthy host response to natural or man-made stresses. However, systemic or runaway inflammation is pathological and it is incumbent to understand better how the inflammatory process is curbed, either naturally or with intervention. This special issue invited submissions to expand our understanding of contemporary mechanisms or approaches to curb the inflammatory response in three broad settings: (1) the systemic inflammatory response, (2) chronic inflammation, and (3) natural pathways in inflammatory resolution. The editors selected a good balance between original research papers and review articles that addressed each area of the call.

2. Curbing the Systemic Inflammatory Response

The systemic inflammatory response was first described in the critical care field for detecting and managing the whole body inflammatory reaction in acutely ill patients to sepsis, burns, or traumatic injuries [1]. It has also been adapted to recognize the iatrogenic triggering of a systemic inflammatory response in heart surgery due to contact activation of blood in the extracorporeal bypass circuit [2].

This special issue leads with three reviews discussing contemporary approaches towards curbing the systemic inflammatory response in sepsis, burn wounds, and neurogenic inflammation (A. M. Bernard and G. R. Bernard; J. A. Farina Jr. et al.; K. M. Lewis). Interleukin- (IL-) 6 and tumor necrosis factor (TNF-) α are identified in sepsis and burns in patients as important mediators at the top of the inflammatory cascade that may be targeted either through specific antibody therapy or through the early excision of full thickness burn tissue that may otherwise act as a cytokine reservoir. The papers echo the PIRO paradigm put forward to integrate how predisposing factors (P), the type of Insult (I) and the host response (R) combine to generate organ injury (O) [3]. This paradigm has helped shift therapeutic strategies towards attenuating the PIR steps before Organ injury (O) occurs. A key transition from PIR to O occurs at the level of endothelial barrier function that serves to protect organs from the inflammatory milieu in the circulation [4]. The loss of blood brain barrier (BBB) function is brought into focus by the paper on neurogenic inflammation, which discusses substance P as a key mediator of BBB permeability and, hence, as an attractive therapeutic target for attenuating injury to the central nervous system following traumatic brain injury, stroke, and meningitis.

Preexisting factors have also been identified in heart surgery to explain the differential inflammatory response in patients towards a common iatrogenic insult [5]. An original paper in this special issue identified high white cell count at preadmission as a predictor of surgical complications, as assessed by frequency of 30-day readmission postsurgery

(J. R. Brown et al.). White blood cell count is considered a simple but valid measure of the prevailing inflammatory status of a patient [6] and, hence, this research again fits the PIRO model of the systemic inflammatory response.

3. Curbing Chronic Inflammation

This special issue attracted some thought-provoking reviews on areas of chronic inflammation that have been somewhat overlooked or less popular in the literature. One review pointed out the surprising decline in interest among researchers for investigating the role of inflammation *after* an acute myocardial infarction (AMI) as opposed to the causal role *prior* to an AMI [7] that is well covered in the literature (P. R. B. Evora et al.). This minority viewpoint, however, chimes with other advocates who have argued consistently that certain nagging gaps remain to be explained in understanding the role of inflammation on AMI outcomes [8, 9]. The review identifies endothelial injury as a gateway in the pathophysiology of ischemic heart disease and presents a conceptual overview for curbing this inflammatory disease process. Transient ischemia is a potential trigger for vascular permeability changes in endothelium and a submission on metabolic acidosis addresses this aspect as part of a strategy to curb inflammation (T. R. de Nadai et al.). Finally, an original paper compared the immunosuppressive properties of statins on T-cell immune responses with other conventional immunosuppressive agents, such as cyclosporine or dexamethasone (A. Jameel et al.). The paper demonstrated differential immunosuppressive properties of statins on T-cell proliferation, IL-1β, IL-17, and interferon- (IFN-) γ production depending on the type of immune activation.

4. Natural Pathways for Inflammatory Resolution

This special issue concludes with two research papers and a review addressing the topical issue of proresolving pathways [10] and how to harness these endogenous pathways to curb inflammation (B. J. Evans et al.; R. C. Landis et al.; T. J. Ahmed et al.). Two papers describe the evolution of the "wound healing" macrophage [11, 12], defined by the expression of CD163 (hemoglobin scavenging receptor), utilizing *in vitro* and *in vivo* approaches. $CD163^+$ macrophages are shown to promote anti-inflammatory and cytoprotective pathways *in vitro* to limit prooxidant injury due to free heme, via pathways involving phosphatidylinositol-3-kinase activation, Akt phosphorylation, and IL-10 secretion. $CD163^+$ cells were associated in the second paper with the phagocytic removal of apoptotic neutrophils, hence limiting potential histotoxic injury, with a shifting of the cytokine profile from proinflammatory mediators, including TNF-α, IL-6, IL-8/CXCL8, monocyte chemoattractant protein (MCP-1/CCL2), macrophage inflammatory protein (MIP1α/CCL3), MIP-1β/CCL4, and eotaxin (CCL11), towards immunoregulatory mediators, including macrophage-derived chemokine (MDC/CCL22), interferon-inducible protein (IP-10/CXCL10), and transforming growth factor (TGF)-β. Finally, an interesting review of proresolving mediators highlights the promise of melanocortin peptides as agents to limit the inflammatory process and protect tissues in a variety of preclinical models for inflammatory disease.

5. Conclusion

The editors are pleased to present this special issue on curbing inflammation and trust it will be popular with a wide readership, from basic scientists, critical care physicians, surgeons, rheumatologists, cardiovascular researchers, and many more, since the inflammatory response impacts on so many fields and disease processes. The editors hope that this special issue will provide a conceptual framework and stimulate new ideas in the development of therapeutic strategies to curb the pathological inflammatory response.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 434586, 11 pages
http://dx.doi.org/10.1155/2013/434586

Research Article

Statin Modulation of Human T-Cell Proliferation, IL-1β and IL-17 Production, and IFN-γ T Cell Expression: Synergy with Conventional Immunosuppressive Agents

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Received 15 March 2013; Accepted 9 July 2013

Academic Editor: David A. Hart

HMG-CoA reductase inhibitors (statins) have been demonstrated to be immunomodulatory for human immune-mediated disease and in experimental models. The aim of this study was to compare statin-mediated immunosuppressive effects on human T-cell responses *in vitro* with those of conventional immunosuppressives (dexamethasone, cyclosporin A (CsA), mycophenolate, and rapamycin). Statins (atorvastatin, lovastatin, and simvastatin) were investigated for their modulatory effects on human PBMC viability, cytokine profiles, and T-cell proliferation. At concentrations that inhibited anti-CD3/28-stimulated T-cell proliferation ($P < 0.01$), simvastatin significantly decreased intracellular $CD4^+$ T-cell expression of IFN-γ ($P < 0.01$) to levels similar to those induced by conventional immunosuppressives. Atorvastatin and lovastatin also decreased IFN-γ expression, although to a lesser degree ($P < 0.05$). All three statins reduced levels of IL-17 production ($P < 0.01$). However, in response to anti-CD3/28 stimulation, simvastatin significantly upregulated IL-1β production ($P < 0.05$). The profile of cytokines produced in response to anti-CD3/28 stimulation was similar when both atorvastatin and dexamethasone were added as compared with dexamethasone alone, suggesting that atorvastatin can synergise with dexamethasone with respect to immunomodulation of cytokines. This data supports the hypothesis of selective statin-mediated immunomodulatory effects on human immune cells.

1. Introduction

As a therapy for hypercholesterolaemia, statins have been used clinically for over two decades. However, over the last decade, immunosuppressive effects have also been demonstrated which are independent of their cholesterol-lowering properties [1]. Statins *in vitro* modulate cell adhesion through effects on endothelial cells and leukocytes, via blocking activation of LFA-1 and decreasing ICAM-1 and MCP-1 expression on activated leukocytes and endothelium [2–4]. Statins have been shown *in vitro* and *in vivo* to reduce leukocyte motility, migration, and infiltration [5]. As compared to cyclosporine, statins were effective in reducing leukocyte infiltration in a rat model of allograft rejection [6]. They also inhibit the NF-κB pathway, involved in transcriptional regulation of cytokines, chemokines, and adhesion molecules [7]. Statins have been shown to upregulate suppressor of cytokine secretion (SOCS) 3 and SOCS 7 which, in turn, downregulate IL-23 and IL-6 production, thus decreasing IL-17 production [8]. Clinical studies of statins in patients with immune-mediated diseases such as rheumatoid arthritis (RA), multiple sclerosis, and organ rejection following transplantation have shown conflicting results due to different statins being used in those studies and often open or retrospective studies involving small patient numbers [9, 10].

Immunomodulatory effects in experimental autoimmune encephalitis (EAE) have been observed with atorvastatin attenuating murine EAE [11], and it was found that atorvastatin upregulated IL-4, IL-5, and IL-10 and downregulated IL-2, IL-12, IFN-γ, and TNF-α. However, in another study investigating atorvastatin in murine experimental autoimmune uveitis (EAU), atorvastatin was found not to modulate

the immune response despite histological grading suggesting mildly decreased inflammation [12], whereas in murine EAU attenuated by lovastatin, there was a decrease in IFN-γ production but no effect on Th2 cytokines [13]. In a Lewis rat model, EAU was decreased in severity by both atorvastatin and lovastatin, even when given after disease onset. With both of these statins, there were decreases in clinical and histological disease scores, antigen responsiveness, and IFN-γ production [14]. Simvastatin was reported to decrease cytokine production, including IL-10, in a murine model of collagen-induced arthritis [15] and in a murine lupus model, it decreased serum TNF-α and IFN-γ levels but increased transcription of IL-4 and IL-10 [16]. Overall, there appear to be a range of immune-related effects by statins depending on species, model, and cell type investigated.

Anti-inflammatory therapy for intraocular inflammation often requires use of corticosteroids, yet these can have severe side effects in the eye including raising intraocular pressure, cataracts, and glaucoma. To reduce these effects, steroid-sparing agents are also used including cyclosporin A (CsA), mycophenolate, and rapamycin. However, these drugs all have systemic side effects which limit their use in the long-term management of chronic disease. In chronic immune-mediated conditions such as RA and systemic lupus erythematosus (SLE), there is an associated increased premature atherogenesis and cardiovascular disease risk secondary to inflammatory processes [17–19]. In addition, patients with uveitis who are treated with steroids and immunosuppressive agents such as cyclosporine and mycophenolate have an increased risk of developing cardiac disease. Therefore, in these cases, the combined cholesterol-lowering and anti-inflammatory properties of statins may be clinically very useful.

The aim of this study was to determine whether individual statins exert immunosuppressive effects on T cells equivalent to those of conventional immunosuppressive agents *in vitro*. The effects of atorvastatin, lovastatin, and simvastatin on normal human whole blood-derived T-cell viability, proliferation, and cytokine responses were studied and compared to dexamethasone, CsA, mycophenolate, and rapamycin. The effects of combining atorvastatin with dexamethasone were also investigated.

2. Materials and Methods

All drugs were dissolved in either dimethylsulphoxide (DMSO), RPMI 1640 (Dutch Modification), and/or 100% ethanol. Atorvastatin (Parke-Davis, Pfizer Inc., NY, USA) was prepared as a 1 mM stock solution; lovastatin (Calbiochem, Nottingham, UK) at 40 mM; simvastatin (MSD, NJ, USA) at 10 μM; dexamethasone (Chauvin, KingstonUpon-Thames, UK) at 400 μg/L.; mevalonate (Sigma-Aldrich Company Ltd, Dorset, UK) at 0.5 M; mycophenolate mofetil (Roche Products Limited, Hertfordshire, UK) at 10 mM; rapamycin (Wyeth, Pfizer, Berkshire, UK) at 0.1 mg/mL; cyclosporin A (Sandoz, Novartis Pharmaceuticals, Surrey, UK) at 1 mg/mL.

2.1. Donors. Peripheral blood was obtained from 16 healthy donors (all working at the Institute/Moorfields) with informed consent (mean age (range), 34.3 (22.5–46.7) years; six males). Exclusion criteria included a history of autoimmune disease, atopy, haematological disorder, or current usage of systemic medication.

The protocols used in this study were reviewed and approved by the Local Ethics Committee. All studies involving human subjects were conducted according to the tenets of the Declaration of Helsinki.

2.2. Reagents. All tissue culture reagents were purchased from Sigma-Aldrich unless otherwise specified. All assays were performed in RPMI 1640 (Dutch Modification) supplemented with 2 mM L-glutamine, 10 μg/mL gentamycin, 20 μM 2-ME, nonessential amino acids, sodium pyruvate, and 10% human AB^+ serum.

2.3. Proliferation and Viability Assays. Peripheral blood mononuclear cells (PBMC) were isolated as previously described [20]. Viability and proliferation assays were performed in triplicate. Cells were incubated for 10 min in a 37°C water bath with 7.5 μM CFSE (Molecular Probes, UK) in serum-free medium. 1 mL cold stop buffer (10% FCS in RPMI) was then added before incubation at room temperature for 30 min. Cells were washed once with RPMI before being resuspended at 2×10^5/mL in T-cell medium for 5 days with or without atorvastatin, lovastatin, simvastatin, rapamycin, mycophenolate, CsA, and dexamethasone. The reversibility of statin function was tested by addition of mevalonate [13]. T cells were stimulated with anti-CD3 (10 ng/mL; clone HIT3a) and anti-CD28 (5 ng/mL; clone CD28.2) antibodies (Pharmingen). All drugs or vehicle controls were added at start of culture, except CsA, which was added 2 h prior to stimulation since no effect was seen with CsA if added at time 0.

Two-color flow cytometry was performed (FACScan; Becton Dickinson, Oxford, UK; BD). Gates were set on viable lymphocytes according to forward (FSC) and side scatter (SSC). Listmode data was generated using CellQuest acquisition software on 15–25,000 events (BD). For viability assays, all lymphocytes were gated to assess level of nonviable propidium iodide expressing (PI^+) cells. For proliferation, nonviable PI^+ cells were excluded.

Data were analysed using WinList (Verity Software House, Topsham, ME). The total numbers of events were determined by analyzing the data using dot plots and rectangular regions to define the cell populations. Histograms were used to track the divisions of CFSE-labeled cells enabling identification of the percentage of divided (proliferated) cells. All data presented are from analyses of live (PI negative) cells only.

2.4. Cytokine Detection. Concentrations of statins required to achieve maximal inhibition of proliferation whilst maintaining viability were identified and used in a multiplex bead array to determine the effects of three statins on cytokine production. 100 μL of heparinised whole blood was cultured with or without simvastatin, lovastatin, atorvastatin, dexamethasone, CsA, mycophenolate, and rapamycin and stimulated with PMA (50 ng/mL) and ionomycin (1 μg/mL). Combinations of dexamethasone and atorvastatin were also

included. Supernatants were harvested at 18 h, centrifuged to remove cells, and stored at −70°C. Multiplex bead cytokine arrays were conducted with a 10-plex kit (Bender Medsystems, Ebiosciences, Hatfield, UK) as per the manufacturer's instructions. Supernatants were analyzed simultaneously for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and TNF-α. The lowest levels of detection were IL-1β (6.2 pg/mL); IL-2 (6.3 pg/mL); IL-4 (5 pg/mL); IL-5 (5.8 pg/mL); IL-6 (14.8 pg/mL), IL-8 (58.6 pg/mL); IL-10 (7.3 pg/mL); IFN-γ (5.7 pg/mL), and TNF-α (8.1 pg/mL). IL-17A was assayed by ELISA (R&D Systems, Abingdon, UK), with a minimum level of detection of 62.5 pg/mL.

2.5. Intracellular Cytokine Expression. Whole blood cells were stimulated with PMA/ionomycin, and 10 μg/mL Brefeldin A was added prior to culture for 18 h in the presence or absence of drugs. $CD3^+$ T cells were stained intracellularly with mouse anti-human IFN-γ-FITC (clone 4S.B3) and rat anti-human IL-10-PE (clone JES3-9D7; both BD) as previously described [20].

2.6. Statistics. All intra-assay comparisons of means were analysed using Student's t-tests and for inter-assay data by Mann-Whitney U tests. Kruskal-Wallis tests were used for multiplex cytokine detection analysis for 6 donors, with nonparametric post hoc comparisons. In experiments with CsA, only 5 donors were included due to lack of available donors. Significance was reached when $P < 0.05$, and this was achieved for several assays, despite relatively small sample sizes.

3. Results

3.1. Effect on T-Cell Viability. To determine whether the drugs had any effect on T-cell viability, cells were cultured in the presence of drugs, mevalonate, and vehicle controls for 72 h prior to staining for PI (Figure 1). A range of concentrations were investigated, and viability was generally high with the lowest viability observed at the highest concentrations of lovastatin and atorvastatin. This was reversed with the addition of mevalonate, a downstream product of HMG-CoA reductase ($P < 0.05$) relative to lovastatin alone, suggesting that these higher drug concentrations reduced cell viability by inhibiting the mevalonate pathway and not via direct toxicity.

3.2. Effect on T-Cell Proliferation. There was a dose-dependent inhibition of T-cell proliferation with all statins, with maximal inhibition at 50 μM atorvastatin and lovastatin and 100 μM simvastatin ($P < 0.01$; Figure 2(A)). The addition of mevalonate, fully restored proliferation (Figures 2(B)–2(E) insert) confirming that these modulatory effects were statin-mediated.

A dose-dependent inhibition of anti-CD3/28-induced T-cell proliferation was also seen for the standard immunosuppressive agents with maximal inhibition at 100 μM mycophenolate mofetil, 100 μM rapamycin, (Figure 2(F)), 100 μg/mL dexamethasone, and 100 ng/mL CsA (data not shown).

3.3. Effects of Statins on Cytokine Production. The three statins demonstrated heterogeneous effects on cytokine production. Six human donors' PBMC were included in this study, and the responses were highly variable. The mean background levels of cytokines secreted by unstimulated cells were subtracted from all wells. The levels secreted by stimulated cells in the absence of exogenous drugs were as follows: TNF-α (30.31 ± 13.74 pg/mL), IFN-γ (0.66 ± 0.47 pg/mL), IL-10 (337.04 ± 374.8 pg/mL), IL-4 (4.47 ± 6.64 pg/mL), IL-1β (447.46 ± 236.07 pg/mL), IL-6 (2086.01 ± 489.49 pg/mL), IL-5 (14.53 ± 13.66 pg/mL), IL-17 (1186.50 ± 599.27), and IL-2 (1.50 ± 2.45 pg/mL). Following stimulation, there was variability in the cytokines produced among the donors, not unexpected in a mixed donor population. Nevertheless, for all six donors, simvastatin significantly increased IL-1β (Figure 3; $P < 0.05$), and all three statins inhibited IL-17 production ($P < 0.01$). Since not all donors' cells exhibited the same cytokine response profile, trends were observed although these failed to reach significance. All three statins caused decreases in levels of IFN-γ and IL-6. Atorvastatin reduced IL-1β, IL-4, IL-5, and IFN-γ and increased IL-10, TNF-α, and IL-2. Lovastatin increased IL-1β and IL-5 production and decreased IL-10, TNF-α, and IFN-γ production. Simvastatin was shown to decrease IL-2, IL-4, and IL-10 production. Mean levels of IFN-γ were relatively low, suggesting a low frequency of IFN-γ secreting cells in the normal donor population.

3.4. Differential Effects of Statins on Cytokine Responses. Due to the variable cytokine response profiles seen in individual donors, we investigated whether there were any correlations between the cytokines that were common to the drugs in comparison with cells stimulated in the absence of drug (PMA/ionomycin alone). Table 1 summarizes the effects on cytokine profiles of the different drugs. All of the correlations included were statistically significant ($P < 0.05$). Stimulation in the absence of statins induced a strong correlation between IL-2 and IFN-γ and between IL-6 and TNF-α. In contrast, following atorvastatin treatment, IL-6 showed a good correlation with IL-2, IFN-γ, and TNF-α, and IL-2 correlated strongly with IFN-γ and TNF-α, as did IFN-γ with TNF-α. In the simvastatin-treated group, IL-6 correlated with IL-2, IFN-γ, and IL-1β and IL-2 with IFN-γ. Finally, the lovastatin-treated group showed correlations of IL-6 with IFN-γ, IL-5 with TNF-α, and IFN-γ with TNF-α.

3.5. Effects of Atorvastatin Combinations with Dexamethasone on Cytokine Responses. Atorvastatin was selected to investigate its effects on cytokine responses in combination with dexamethasone. In the presence of either drug alone or when added in combination, IL-6, IL-8, IFN-γ, and IL-1β levels were all decreased although these did not reach significance, suggesting that the immunosuppressive effects of each of the drugs were maintained when added in combination (Figure 4). Interestingly, the level of IL-10 production was enhanced in the presence of dexamethasone, although this failed to reach significance. For the production of IL-2, IL-5, and TNF-α, the effects of atorvastatin were reversed upon addition of dexamethasone, or with dexamethasone alone, suggesting that this drug combination was antagonistic

Table 1: Spearman rank analysis of correlations in cytokines produced by cells cultured with PMA/ionomycin ± statins ± dexamethasone. The values represent the strength of the correlation of cytokines, with +1.00 being the maximum, and their changes in expression according to exposure to each statins. All results were statistically significant ($P < 0.04$).

	No drug		**Atorvastatin**			**Simvastatin**			**Lovastatin**			**Dex**		**Atorvastatin and dex**		
	IFNγ	TNFα	IL-2	IFNγ	TNFα	IL-1b	IL-2	IFNγ	IL-5	IFNγ	TNFα	IL-5	IFNγ	IL-1β	IFNγ	TNFα
IL-2	+0.89		■	+1.00	+0.99		■	+0.89				−0.94	+0.94			
IL-6		+0.89	+0.89	+0.89	+0.93	+0.94	+1.00	+0.89	+0.94	+0.89	+0.99			+0.94		
IFNγ	■			■	+0.99			■		■	+0.99	−0.89	■		■	
IL-10																+0.89

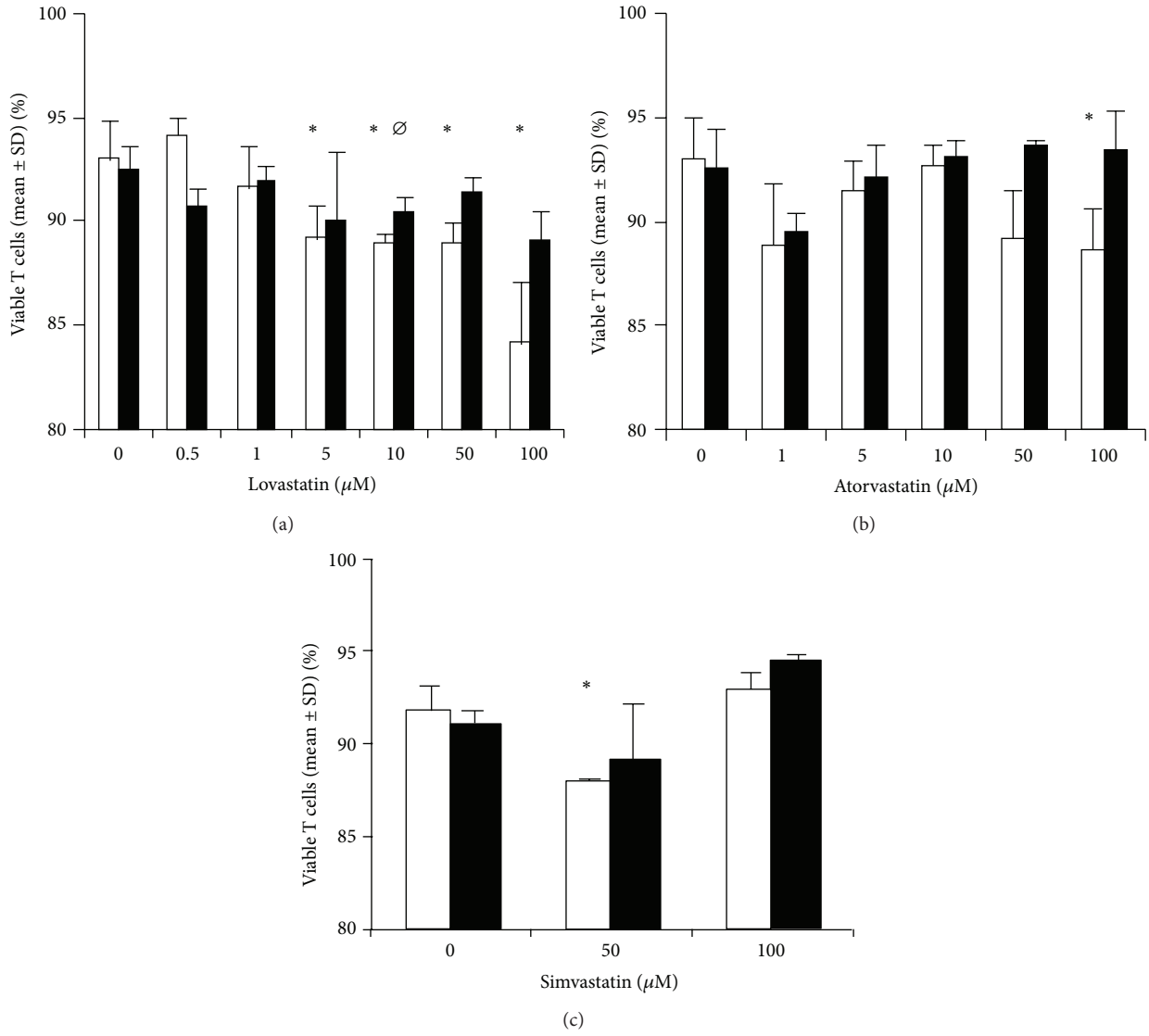

Figure 1: Cell viability was assessed at all concentrations of statins. The percentage viability is compared in the presence (filled bars) or absence of mevalonate (clear bars). ∗ denotes a statistical difference between viability of cells in different concentrations of statins ($P < 0.05$), and ∅ indicates a difference in viability with and without mevalonate ($P < 0.05$).

with respect to some cytokine responses. For the cytokine correlations (Table 1), there was a baseline correlation between IL-6 and TNF-α following addition of atorvastatin but not in the presence of dexamethasone alone or combined with atorvastatin. In contrast, the correlation of IL-2 with IFN-γ was maintained in the presence of either atorvastatin or dexamethasone alone but was lost when the drugs were added in combination. This suggests differential modulatory effects, with the combination of dexamethasone and atorvastatin exerting a distinct pattern of cytokine responses.

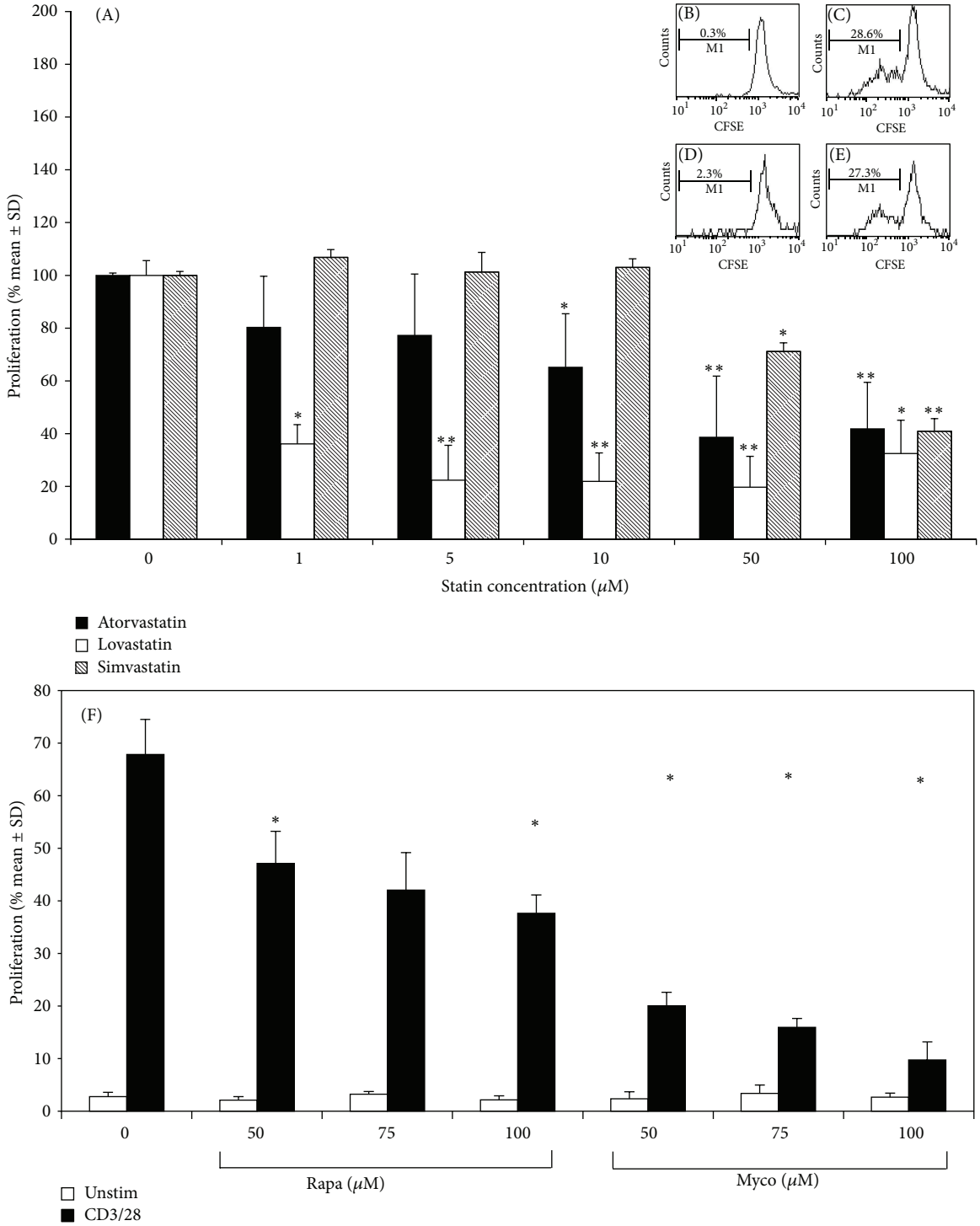

Figure 2: (A)–(F). Effect of statins on αCD3/αCD28-stimulated T-cell proliferation. Each data point represents the mean % T-cell proliferation ± SD. Due to intersample variation, data from each experiment ($n \geq 3$) were normalized against the positive control of % proliferated T cells without drug, allowing experiments to be pooled ($n \geq 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to control. Insert: one representative FACS analysis is shown. (A), Control histogram showing % divided T cells (M1) in the absence of stimulation or statin. (B), % divided T cells after stimulation but no statin. (C), % divided T cells with the addition of atorvastatin (50 μM) as compared to stimulated control. (D), % divided T cells with the addition of atorvastatin (50 μM) and mevalonate (200 μM). (F), Effect of rapamycin and mycophenolate on αCD3/αCD28-stimulated T-cell proliferation. Each data point represents the mean % change in T-cell proliferation ± SD. Due to intersample variation, data from each experiment ($n \geq 3$) were normalized against the positive control of % proliferated T cells without drug, allowing experiments to be pooled ($n \geq 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to control.

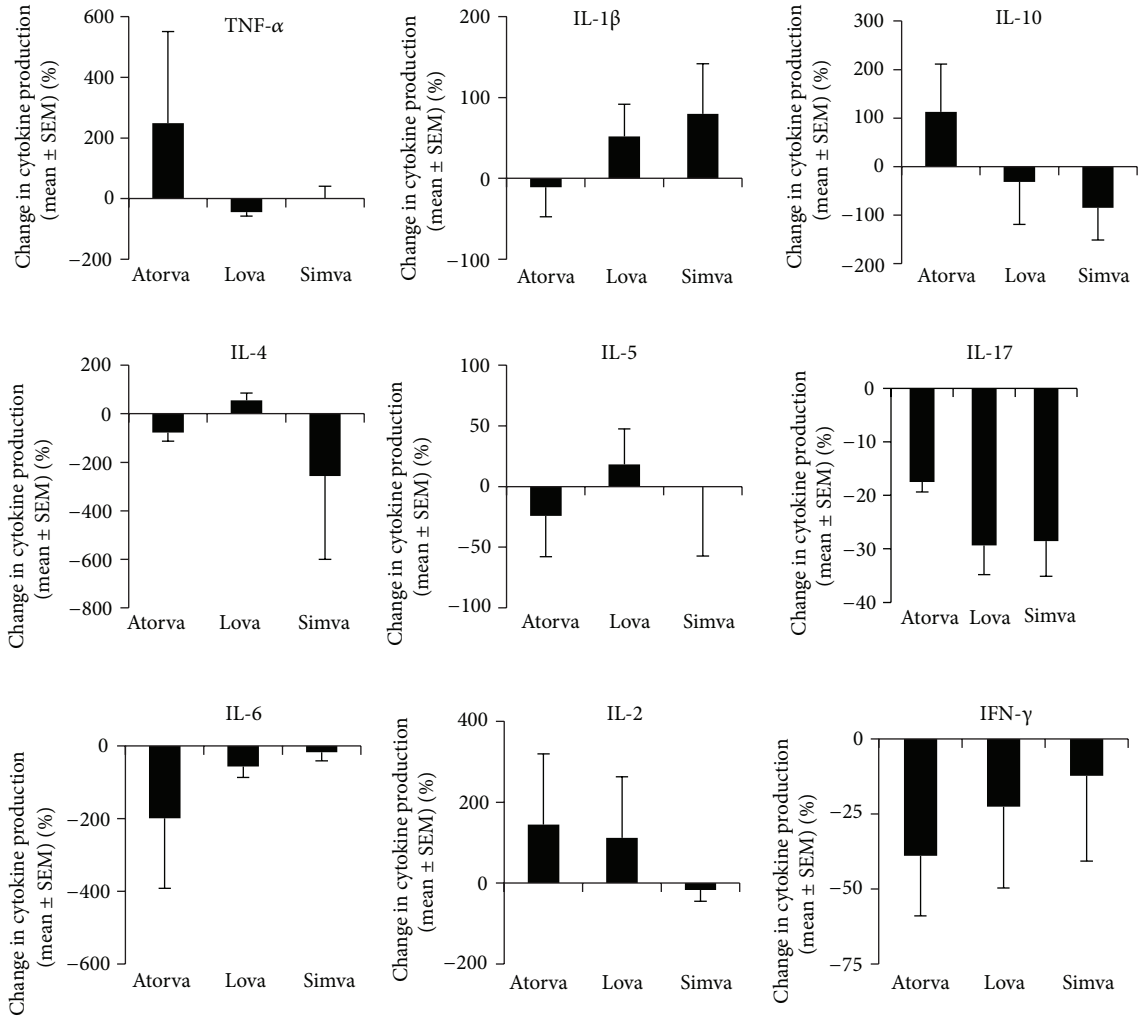

Figure 3: Graphs showing percentage changes in cytokine production by cells cultured with PMA/ionomycin ± statins. These studies show a multitude of varied responses indicating that the different statins have slightly different profiles of action.

3.6. Effect of Immunosuppressive Drugs and Statins on Intracellular IFN-γ Expression. For a more sensitive method of detecting IFN-γ, intracellular cytokine staining was performed. There was a significant decrease in intracellular expression of IFN-γ in $CD3^+$ T cells in the presence of the statins ($P < 0.05$ for atorvastatin and lovastatin; $P < 0.01$ for simvastatin). Dexamethasone, CsA, mycophenolate, and rapamycin also demonstrated significant decreases in intracellular IFN-γ expression as compared to stimulated controls ($P < 0.01$; Figure 5). Dexamethasone exerted the most significant reduction in expression of intracellular IFN-γ, whilst the combination of dexamethasone with atorvastatin also significantly reduced IFN-γ ($P < 0.05$).

4. Discussion

Statins have been demonstrated to exert anti-inflammatory effects *in vitro*: reduction in CD11b expression [21]; inhibition of IFN-γ-induced MHC-II expression on endothelial cells, macrophages, and therefore T-cell activation [22]; disruption of lipid rafts essential for T-cell activation [23]. Considering the use of statins as an adjunctive therapy for relapsing remitting multiple sclerosis, the 2010 Cochrane review on "Statins for multiple sclerosis" concluded that data from double-blinded randomized trials would be needed before licensing for treatment of multiple sclerosis [24]. In spite of the large amount of *in vitro* data indicating a potential anti-inflammatory role of statins, there is little evidence for their direct role in modulating human T-cell cytokines.

The concentrations of statins required to inhibit T-cell responses to anti-CD3/28 stimulation used for this *in vitro* study were selected from titration experiments, and these concentrations have been used in other *in vitro* studies [25–27]. Due to the potency of the *in vitro* stimulants, higher, nonphysiological concentrations of statins were required, and therefore, we cannot extrapolate the data to statin doses used clinically [28]. Nevertheless, these studies can inform on the effects of statins on human T-cell responses. Comparing the effects of drugs on T-cell proliferation, the statins inhibited proliferation to a comparable level to that achieved with conventional immunosuppressive drugs. The viability of the cells in our study was reduced in the presence

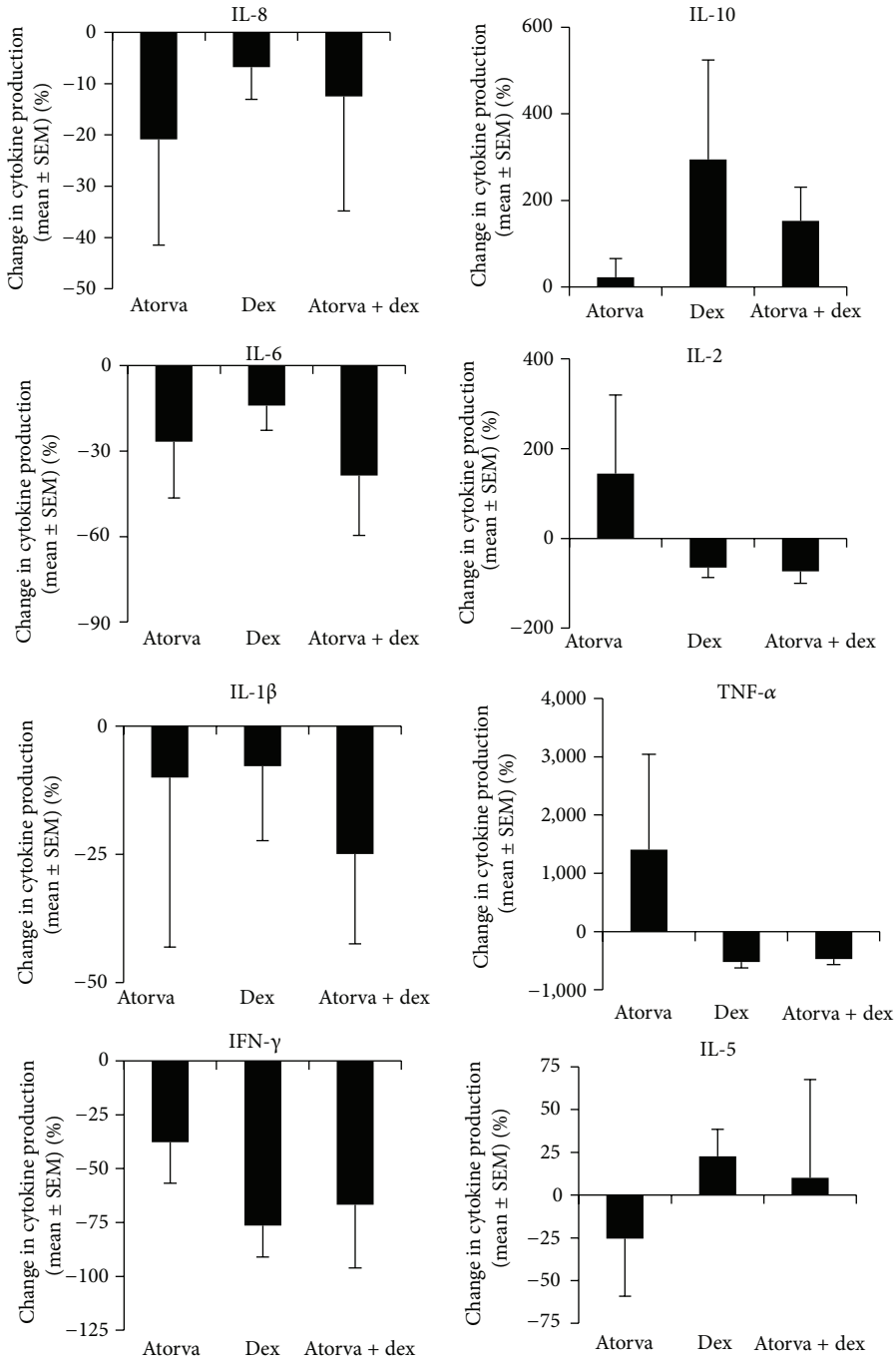

Figure 4: Graphs showing percentage changes in cytokine production in cells stimulated with PMA/ionomycin ± atorvastatin/dexamethasone.

of these statin concentrations, but, following the addition of mevalonate, there was a recovery both in viability and in proliferation, confirming that the inhibitory effect was reversible, and a direct result of statin-mediated inhibition of the HMG-CoA pathway.

Of the conventional immunosuppressive drugs investigated, dexamethasone exerted the most potent immunomodulatory effect, and its ability to decrease production of IL-1β, IL-2, IL-6, IL-8, IFN-γ, and TNF-α is in agreement with previous studies [29, 30]. Interestingly, dexamethasone treatment upregulated IL-10 production, as reported by others [31]. Of course the study design does not permit us to determine the cellular source of the cytokines, and monocytes, which were likely to be present at low numbers, have previously been observed to respond to simvastatin by upregulating IFN-γ production which, in turn, led to a decrease in IL-17 production [8]. The downregulatory effect of other immunosuppressive drugs (CsA, rapamycin, and

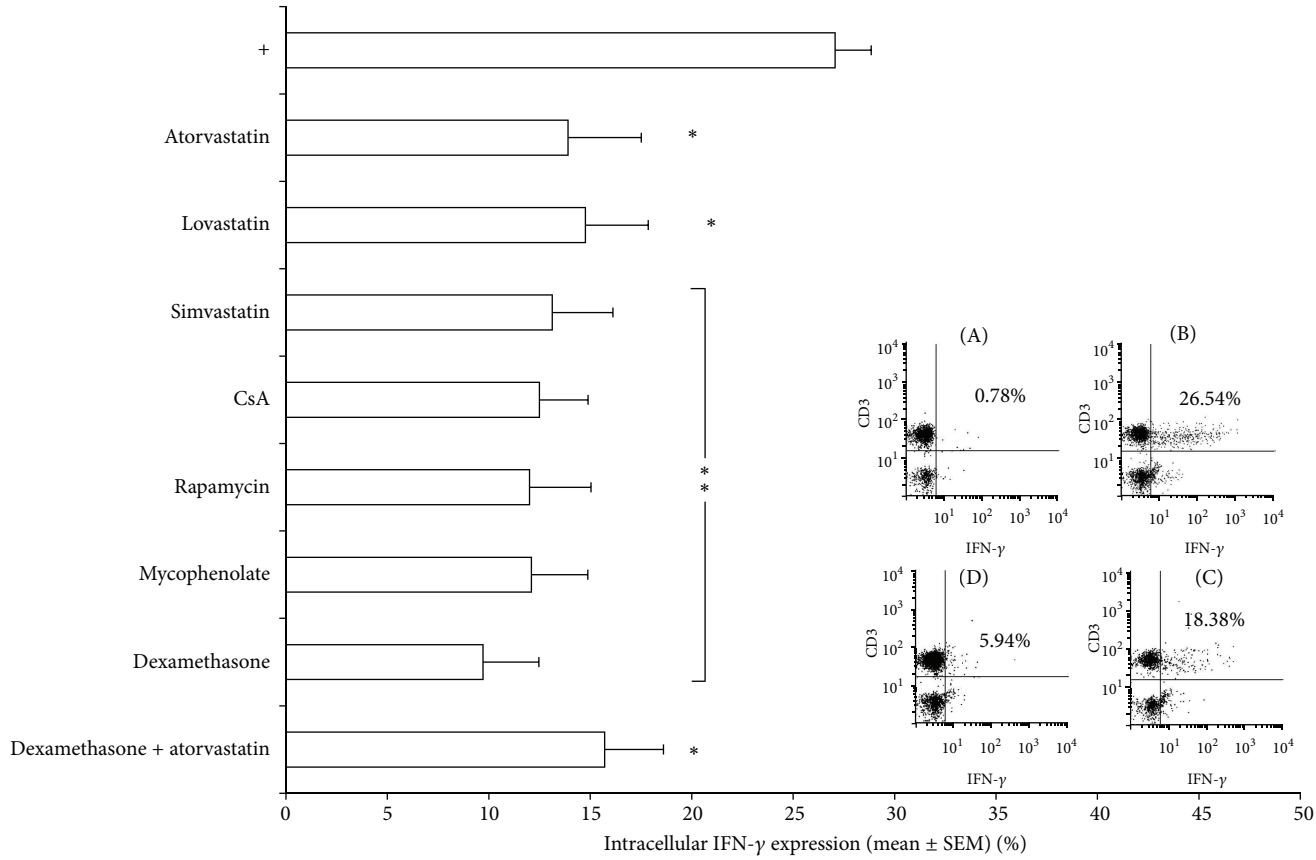

Figure 5: Percentage of IFN-γ expression in PMA/ionomycin stimulated whole blood with or without addition of drugs. Each bar represents the means ± SEM from 6 experiments, each performed in triplicate, subtracting basal cytokine expression. Drug concentrations: atorvastatin (50 μM), lovastatin (50 μM), simvastatin (100 μM), rapamycin (100 μM), mycophenolate (100 μM), CsA (100 ng/mL), and dexamethasone (400 μg/mL). $^{*}P < 0.05$; $^{**}P < 0.01$. One representative FACS analysis is shown. (A), Unstimulated $CD3^{+}$ T cells showing basal IFN-γ expression. (B), Stimulated $CD3^{+}$ T cells to demonstrate baseline levels of IFN-γ expression. (C), Stimulated $CD3^{+}$ T cells treated with atorvastatin (50 μM) demonstrating a decreased level of IFN-γ expression as compared to control. (D), Stimulated $CD3^{+}$ T cells treated with dexamethasone (400 μg/mL), showing a decreased level of IFN-γ as compared to group (C).

mycophenolate) on IFN-γ expression is in agreement with other studies [29, 32–34].

These drugs also differentially inhibited other cytokines, with CsA significantly decreasing levels of IL-2, IL-4, and IL-5 IFN-γ but not IL-10 production (data not shown). In contrast, a decrease in IL-10 mRNA expression has been reported in a study on CsA-treated normal human whole blood following anti-CD3/CD28 T cell costimulation [29]. However, changes in IL-10 mRNA expression do not necessarily correlate with the level of protein production, which could explain the contradictory findings. Mycophenolate has recently been demonstrated to have a profound inhibitory effect on Th17 cells [35], and we have not yet investigated this drug for its effects on Th17 cells in our model (in progress). Other discrepancies in cytokine responses observed across the literature are easily explained by the different genetic backgrounds of donors used in different studies.

All three statins in this study significantly reduced intracellular IFN-γ expression, as previously reported for mouse and human T cells [15, 36, 37]. For the other cytokines investigated in this study, there were no effects common to all three statins. Intracellular expression levels of IL-2, IL-4, and IL-10 were not significantly altered (data not shown) due to the variable responses between the individual donors and very low levels of IL-4 and IL-10 expression.

Atorvastatin, when given to hypercholesterolemic patients, has been found to decrease PBMC production of TNF-α IL-1, IL-6 [38], IL-2, and IFN-γ *in vitro* [39]. Decreased serum IL-8 and IL-6 levels have been observed with atorvastatin postcoronary artery bypass grafts [40, 41]. More recently, atorvastatin has been reported to reduce the pathogenic production of IL-6 and IL-10 by activated T cells from SLE patients thereby readjusting them toward a more tolerant phenotype [42]. In our study, atorvastatin decreased production of IL-17 and intracellular expression of IFN-γ, in agreement with data from a Lewis rat model of experiment autoimmune neuritis [37] and in Balb/c mice with experimental colitis, in which TNF-α was also downregulated [43].

In murine EAE treated with lovastatin, reduced levels of IL-6, TNF-α, and IFN-γ with upregulation of IL-4, IL-5, and IL-10 were found, suggesting a Th2 polarization [44, 45]. We

detected significant decreases in production of IL-17 and in IFN-γ expression in response to lovastatin.

Simvastatin decreased IL-17 and IFN-γ expression and increased IL-1β. It has previously been found that simvastatin decreased TNF-α in hypercholesterolemic patients [46] but had inconsistent effects on plasma IL-6 levels [47]. Simvastatin was not found to influence TNF-α, IL-6, and IL-1 receptor antagonist levels in an endotox in induced human *in vivo* model of low-grade inflammation [48] but decreased IL-8 production by PBMCs of CAD patients after 6 months of systemic treatment [49]. This contrasts with *in vitro* effects of simvastatin on anti-CD3/anti-CD28-stimulated PBMCs from RA patients with decreased IFN-γ and IL-10 unaffected [15]. Simvastatin also decreased levels of serum IL-6 in mouse collagen-induced arthritis [15] and IL-17 gene expression in human MS patients $CD4^+$ T cells [8].

Due to the clinical use of atorvastatin for controlling cholesterol levels, it was selected in this study for possible combinatory effects when added with dexamethasone. However, by comparing atorvastatin, dexamethasone, and dexamethasone/atorvastatin together, no significant differences were found in overall levels of cytokines produced. However, the combination did affect cytokine correlations, suggesting that there might be an additive anti-inflammatory effect. There is little in the literature to suggest contraindications in terms of side effects, and the combination has been used experimentally in a rat model to reverse dexamethasone-induced hypertension [50].

In conclusion, different statins, with their distinct effects on cytokine responses, could have clinical applications in specifically targeting those key cytokines relevant to each inflammatory disease process, perhaps as a corticosteroid-sparing therapy, while providing effective disease control.

Abbreviations

CsA:	Cyclosporine A
HMG Co-A:	3-Hydroxy-3-methyl-glutaryl coenzyme A
EAE:	Experimental autoimmune encephalitis
EAU:	Experimental autoimmune uveitis
PI:	Propidium iodide.

Conflict of Interests

This is to confirm that none of the authors had any conflict of interests during the undertaking of this research study and during the preparation of this paper. Where it was necessary to purchase reagents from commercial sources for this study, those companies are named herein.

Funding

This work was partly funded by the Faculty of Health Sciences, University of Adelaide, Australia (KO).

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 781024, 7 pages
http://dx.doi.org/10.1155/2013/781024

Research Article

Preoperative White Blood Cell Count and Risk of 30-Day Readmission after Cardiac Surgery

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Received 24 April 2013; Revised 26 June 2013; Accepted 26 June 2013

Academic Editor: Paulo Roberto Barbosa Evora

Approximately 1 in 5 patients undergoing cardiac surgery are readmitted within 30 days of discharge. Among the primary causes of readmission are infection and disease states susceptible to the inflammatory cascade, such as diabetes, chronic obstructive pulmonary disease, and gastrointestinal complications. Currently, it is not known if a patient's baseline inflammatory state measured by crude white blood cell (WBC) counts could predict 30-day readmission. We collected data from 2,176 consecutive patients who underwent cardiac surgery at seven hospitals. Patient readmission data was abstracted from each hospital. The independent association with preoperative WBC count was determined using logistic regression. There were 259 patients readmitted within 30 days, with a median time of readmission of 9 days (IQR 4–16). Patients with elevated WBC count at baseline (10,000–12,000 and >12,000 mm^3) had higher 30-day readmission than those with lower levels of WBC count prior to surgery (15% and 18% compared to 10%–12%, $P = 0.037$). Adjusted odds ratios were 1.42 (0.86, 2.34) for WBC counts 10,000–12,000 and 1.81 (1.03, 3.17) for WBC count > 12,000. We conclude that WBC count measured prior to cardiac surgery as a measure of the patient's inflammatory state could aid clinicians and continuity of care management teams in identifying patients at heightened risk of 30-day readmission after discharge from cardiac surgery.

1. Introduction

Approximately one in every five hospitalized patients is readmitted within 30 days [1]. Currently, two-thirds of US hospitals have reimbursement penalties for higher than expected 30-day readmission rates from the Center for Medicaid and Medicare Services [2, 3]. It is expected that similar penalties will be extended to other procedures and diagnoses including cardiac surgery. In preparation for the expansion of the penalty system in the USA and to improve prediction of patients at high risk of postdischarge complications leading to readmissions or premature death, risk factors must be

identified early in the hospital course to align the best possible quality and continuity of care.

Currently, a validated risk model for predicting readmissions after cardiac surgery is not available and few risk factors for readmission are known. Recent evidence from California reported an association between infection and higher rates of 30-day readmission after cardiac surgery [4]. However, identification of infection after discharge without routine monitoring of a postcardiac surgical patient is problematic. What is needed is for clinical care teams to identify patients at high risk of infection before cardiac surgery to determine readiness and safety for the patient to undergo surgery. A common marker of inflammation is white blood cell (WBC) count, routinely measured prior to cardiac surgery. WBC count provides a broad measure of inflammation status, whether as a result of infection or proinflammatory disease states such as diabetes, COPD, or hemodialysis [5–8]. Elevated WBC count is reported as a component of the systemic inflammatory response syndrome (SIRS) to sepsis and is endorsed as a marker for reporting the systemic inflammatory response to cardiopulmonary bypass [9, 10]. In addition, current evidence has shown that preoperative WBC count is predictive of in-hospital mortality and stroke [11] and major bleeding [12] after coronary artery bypass graft surgery and associated with complications in other endovascular and thoracic procedures [13, 14], suggesting that preoperative WBC count may aid clinical care teams in risk-stratifying patients prior to surgery. However, it is not known if a patient's baseline inflammatory state measured by crude WBC count could predict 30-day readmission. Therefore, we sought to evaluate whether preoperative WBC count was associated with 30-day readmissions after cardiac surgery.

2. Methods

Patients undergoing coronary artery bypass graft (CABG) surgery and/or valve surgery within the Northern New England Cardiovascular Disease Study Group (NNE) between July 2008 and December 2010 were enrolled in the cohort. A total of 2,209 consecutive patients were included along with 268 readmissions to the hospital performing the index cardiac surgery. Twelve patients were excluded due to missing white blood cell counts and twenty-one for incomplete data, leaving a total of 2,176 patients and 259 readmissions occurring within 30 days of discharge from the index cardiac surgery admission. All institutional review boards for each center reviewed and approved the data collection for the NNE registry and supplementary data collection for readmissions.

The NNE is a voluntary regional consortium of physicians, allied health professionals, research scientists, and hospital administrators from institutions in Maine, New Hampshire, and Vermont that support coronary revascularization and open-heart surgery. The goal of the consortium is to foster continuous improvement in the quality, safety, and effectiveness of care for patients with cardiovascular disease through the analysis of process and outcomes data with timely feedback to the health care professionals providing these services. All the hospitals providing open-heart surgery in this region contribute data on consecutive cases with validation of procedure numbers and mortality performed every two years. The registry collects data on patient characteristics, procedural indication, priority, and process, and in-hospital outcomes (see http://www.nnecdsg.org/ for the data forms and publically available data).

WBC count was defined as the last preoperative measurement of WBC taken prior to procedure, was collected by data abstractor at each center. Categories of WBC counts were

Table 1: Characteristics of patients with or without 30-day readmission.

Characteristic	30-day readmission		
	No	Yes	*P* value
Number of patients (2,176)	1,917	259	
Demographics			
Age	66.2 ± 11.2	66.4 ± 11.5	0.740
Female	28.5	32.8	0.154
BMI	29.6 ± 5.9	29.8 ± 6.5	0.585
Comorbidities			
Type 2 diabetes mellitus	31.5	37.1	0.070
Vascular disease	27.3	30.9	0.224
COPD	15.6	20.5	0.043
History of dialysis	2.4	5.0	0.015
Smoking	21.4	22.4	0.726
Cardiac history			
Recent MI	17.3	13.1	0.091
CHF	19.9	25.1	0.051
Prior CABG	3.9	2.7	0.337
Prior valve	1.5	1.9	0.611
Prior PCI	18.2	17.4	0.760
NYHA Class IV	15.6	15.4	0.949
Cardiac anatomy and function			
Left main disease ≥50%	28.8	20.9	0.007
Single-vessel disease	33.5	42.6	0.002
Two-vessel disease	28.5	30.2	
Three-vessel disease	38.1	27.3	
Ejection fraction			
<40%	11.3	12.3	0.894
40%–59%	12.6	11.1	
50%–59%	24.3	24.2	
≥60%	51.7	52.4	
White blood cell count (in 1,000's, mm^3)			
<6.0	19.9	19.7	0.037
6.0–7.9	39.2	35.9	
8.0–9.9	25.3	21.6	
10.0–12.0	9.9	13.1	
>12.0	5.6	9.7	

COPD: chronic obstructive pulmonary disease; MI: myocardial infarction; CHF: congestive heart failure; CABG: coronary artery bypass graft surgery; PCI: percutaneous coronary intervention; WBC: white blood cell; eGFR: estimated glomerular filtration rate.

Table 2: Procedural characteristics and outcomes of patients with or without 30-day readmission.

Characteristic	30-day readmission		
	No	Yes	*P* value
Procedural characteristics			
Priority			
Emergent	5.3	6.6	0.534
Urgent	50.9	47.9	
Elective	43.9	45.6	
Procedure			
CABG	60.7	51.0	0.007
Valve	22.4	30.1	
CABG/valve	17.0	18.9	
On-pump surgery	90.3	95.8	0.004
Nadir hematocrit <20 on bypass	15.0	20.5	0.029
Cardiopulmonary bypass time (min)	119.7 ± 53.7	122.9 ± 56.5	0.393
Time to initial extubation (min)	17.3 ± 65.2	17.6 ± 35.1	0.951
Intraoperative myocardial infarction	2.4	2.7	0.725
Return to bypass	4.2	4.6	0.730
Management			
RBC transfusions			
None	67.6	58.7	0.001
One	8.9	10.0	
Two	10.3	9.3	
Three or more	13.2	22.0	
Use of 1 or more inotropes			
Arrive to ICU	43.9	44.0	0.965
After 4 hours	37.2	44.0	0.034
After 48 hours	11.3	15.1	0.080
Adverse outcomes			
Low-cardiac output failure	8.0	10.0	0.272
Stroke	1.1	1.5	0.525
Mediastinitis	0.4	2.7	<0.001
Acute kidney injury	30.7	49.4	<0.001
Reintubation	3.7	3.9	0.866
Return to operating room for bleeding	3.2	4.3	0.395
New atrial fibrillation	32.6	39.8	0.021
Leg wound infection	0.7	1.2	0.463
Pneumonia	1.7	0.8	0.275

CABG: coronary artery bypass graft surgery; RBC: packed red blood cell transfusion; ICU: intensive care unit.

divided into predefined categories (<6.0, 6.0–7.9, 8.0–9.9, 10.0–12.0, and >12.0 thousands per cubic millimeter, mm^3).

Baseline, operative, and postoperative outcomes were compared using chi-square tests and continuous data using Student's *t*-test or Wilcoxon rank sum tests where appropriate. We conducted both univariate and backwards stepwise logistic regression removing risk factors that did not reach an alpha <0.1 among only risk factors with an alpha <0.1 from univariate comparisons. All risk factors meeting an alpha <0.1 were included in the final model multivariate logistic regression model. Categories of white blood cell counts were then added to the multivariate clinical risk prediction model. We conducted a Hosmer-Lemeshow goodness of fit test and calculated the area under the receiver operating characteristic (ROC) curve for the final multivariate model with categories of white blood cell count and reported the ROC and 95% confidence intervals for each model. All analyses were performed using Stata 11.2 (College Station, TX).

3. Results

Among the 2,176 patients, 259 patients were readmitted within 30 days (11.9%). The median time of readmission was 9 (IQR 4–16) days. Patient demographics were similar between patients with a 30 day readmission and those without a readmission. Patients readmitted within 30 days were more likely to have chronic obstructive pulmonary disease,

Table 3: Univariate and multivariate regression anaylsis for 30-day readmission.

	Odds ratios (95% CI) for 30-day readmission	
	Univariate	Multivariate
White blood cell count (in 1,000's, mm^3)		
<6.0	Reference	Reference
6.0–7.9	0.93 (0.64, 1.33)	0.96 (0.65, 1.41)
8.0–9.9	0.86 (0.58, 1.29)	0.91 (0.59, 1.39)
10.0–12.0	1.34 (0.84, 2.14)	1.42 (0.86, 2.34)
>12.0	1.73 (1.03, 2.93)	1.81 (1.03, 3.17)
Other risk factors		
Single-vessel disease	1.77 (1.28, 2.46)	1.73 (1.24, 2.43)
Two-vessel disease	1.48 (1.04, 2.10)	1.43 (1.00, 2.06)
On-pump surgery	2.43 (1.31, 4.54)	1.85 (0.98, 3.52)
Nadir hematocrit on bypass <20	1.55 (1.10, 2.17)	1.39 (0.96, 2.00)
Three or more packed red blood cells	1.86 (1.34, 2.56)	1.52 (1.07, 2.18)
Mediastinitis	7.58 (2.64, 21.79)	5.81 (1.87, 18.08)
Acute kidney injury	2.21 (1.70, 2.87)	2.03 (1.53, 2.68)
Model parameters		
Hosmer-Lemeshow χ^2, P value		$\chi^2 = 10.94$, P value = 0.2
ROC		0.66

WBC: white blood cell; SD: standard deviation of the log-transform of WBC count; ROC: area under the receiver operating characteristic curve.

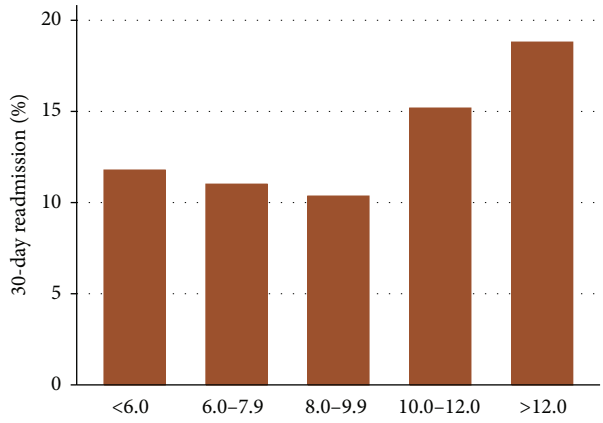

Figure 1: Preoperative White Blood Cell Counts and Risk of 30-day Readmission. The graph plots the risk of all-cause 30-day readmission by five pre-defined categories of preoperative white blood cell counts (in thousands per cubic millimeter, mm^3).

history of dialysis, single vessel coronary disease, and white blood cell counts greater than ten thousand prior to surgery (Table 1). Procedural factors associated with 30-day readmission included valve or combined CABG/valve procedure, on-pump surgery, nadir hematocrit <20 on bypass, three or more packed red blood cell transfusions, use of inotropes, and the development mediastinitis, AKI, or atrial fibrillation (Table 2).

Patients with elevated WBC counts at baseline (10,000–12,000 and >12,000 mm^3) had higher 30-day readmission than those with lower WBC counts prior to surgery (15% and 18% compared to 10%–12%, $P = 0.037$, Figure 1). After backwards stepwise regression, WBC count and other risk factors remained significantly associated with 30-day readmission including number of diseased vessels, on-pump surgery, nadir hematocrit <20 on bypass, receiving three or more packed red blood cells, developing mediastinitis, and acute kidney injury (Table 3). Type of surgery (valve, isolated coronary artery bypass graft, or combined valve/graft) and duration of bypass were not significantly associated with readmissions in the multivariate model. Adjusting odds ratios for preoperative WBC counts were 1.42 (0.86, 2.34) for counts 10,000–12,000 (mm^3) and 1.81 (1.03, 3.17) for counts >12,000 (mm^3) (Table 2). The calculated c-statistic was 0.66 with a Hosmer-Lemeshow goodness of fit chi-square of 10.94 and P value of 0.2. Patient and procedural characteristics stratified by white blood cell categories are summarized in Table 4.

4. Discussion

We explored the predictive ability of WBC counts prior to cardiac surgery on 30-day readmission. With and without adjustment of other risk factors for readmission, patients with preoperative WBC counts >12,000 (mm^3) were significantly more likely to be readmitted to the hospital within 30 days from discharge. We are the first to demonstrate that a marker of inflammation prior to the start of surgery demonstrates increased risk of 30-day readmission and should be incorporated into risk models to predict readmission prior to discharge from cardiac surgery.

WBC count has enjoyed a resurgence in recent years as a valid marker of inflammation and as a strong independent predictor of future coronary heart disease and stroke [15, 16]. After an acute event, patient outcomes remain influenced by WBC count at the time of hospital admission. In several studies, peak WBC count or elevated monocyte count has been

Table 4: Characteristics of patients and white blood cell count categories.

Characteristic	White blood cell count (in 1,000s)					
	<6.0	6.0–7.9	8.0–9.9	10.0–12.0	>12.0	*P* value
Number of patients (2,176)	433	845	541	224	133	
Demographics						
Age	67.7 ± 11.3	67.1 ± 10.6	65.5 ± 11.4	63.6 ± 11.7	63.5 ± 12.1	<0.001
Female	30.3	29.5	26.6	31.7	27.8	0.594
BMI	28.5 ± 5.3	29.6 ± 6.0	30.3 ± 6.2	30.3 ± 6.1	29.4 ± 5.5	<0.001
Comorbidities						
Type 2 diabetes mellitus	26.3	31.6	36.8	32.6	34.6	0.014
Vascular disease	25.6	27.9	27.9	29.9	28.6	0.817
COPD	12.2	14.7	19.4	20.1	18.1	0.009
History of dialysis	2.1	2.4	2.6	2.7	7.5	0.013
Smoking	9.7	17.9	28.8	35.7	30.1	<0.001
Cardiac history						
Recent MI	7.6	15.5	17.6	28.1	33.1	<0.001
CHF	18.7	18.7	21.4	22.3	30.8	0.017
Prior CABG	5.1	3.9	3.1	1.8	4.5	0.256
Prior valve	2.5	1.3	0.9	1.3	3.0	0.173
Prior PCI	15.9	18.6	19.2	17.0	18.8	0.699
NYHA Class IV	12.0	12.2	16.6	24.6	29.3	<0.001
Cardiac anatomy and function						
Left main disease ≥50%	22.9	27.9	27.9	31.3	37.6	0.012
Single-vessel disease	41.6	34.7	33.0	28.1	26.8	0.013
Two-vessel disease	27.5	28.2	28.8	32.9	28.5	
Three-vessel disease	30.9	37.2	38.2	39.1	44.7	
Ejection fraction						
<40%	9.4	9.3	12.0	15.0	22.8	<0.001
40%–59%	11.1	10.9	14.1	18.2	9.5	
50%–59%	25.9	24.2	24.1	21.5	26.0	
≥60%	53.5	55.5	49.8	45.3	41.7	
Procedural characteristics						
Priority						
Emergent	1.6	2.8	6.1	10.3	23.3	<0.001
Urgent	46.0	47.9	51.2	61.2	60.9	
Elective	52.4	49.2	42.7	28.6	15.8	
Procedure						
CABG	50.6	59.1	62.5	67.4	66.2	<0.001
Valve	31.4	23.7	21.3	15.2	16.5	
CABG/valve	18.0	17.3	16.3	17.4	17.3	
On-pump surgery	92.4	92.0	88.7	89.7	90.2	0.213
Nadir hematocrit <20 on bypass	11.8	15.0	13.7	12.1	15.8	0.465
Cardiopulmonary bypass time (min)	121.5 ± 55.2	117.1 ± 49.2	122.1 ± 55.7	121.4 ± 62.2	124.7 ± 57.9	0.656
Time to initial extubation (min)	14.4 ± 28.6	15.6 ± 32.7	16.6 ± 51.4	20.8 ± 51.8	35.9 ± 199.8	<0.001
Intraoperative myocardial infarction	1.2	2.5	2.4	3.1	4.5	0.203
Return to bypass	5.1	4.7	3.0	3.1	5.3	0.343
Management						
RBC transfusion	36.3	32.9	30.7	31.7	43.6	0.041
Use of 2 or more inotropes within 48 hours	2.1	3.6	2.5	4.5	4.6	0.278

Table 4: Continued.

Characteristic	White blood cell count (in 1,000s)					
	<6.0	6.0–7.9	8.0–9.9	10.0–12.0	>12.0	*P* value
Adverse outcomes						
Low-cardiac output failure	7.4	8.8	6.7	8.5	14.3	0.063
Stroke	1.2	0.8	1.5	1.8	0.8	0.687
Mediastinitis	0.2	0.7	0.6	0.9	1.5	0.552
Acute kidney injury	29.3	33.6	31.4	37.5	38.4	0.128
Reintubation	3.0	2.8	4.8	4.5	5.3	0.239
Return to operating room for bleeding	6.0	3.1	1.9	2.2	4.5	0.005
New atrial fibrillation	32.1	34.8	31.8	32.6	36.8	0.650
Leg wound infection	0.7	0.8	0.7	1.3	0.0	0.731
Pneumonia	1.6	1.4	1.1	3.1	1.5	0.354

COPD: chronic obstructive pulmonary disease; MI: myocardial infarction; CHF: congestive heart failure; CABG: coronary artery bypass graft surgery; PCI: percutaneous coronary intervention; WBC: white blood cell; eGFR: estimated glomerular filtration rate; CABG: coronary artery bypass graft surgery; RBC: red blood cell; ICU: intensive care unit.

linked to death or major adverse cardiac events (MACEs) outcomes, including readmission [17–19]. Other strong evidence has linked high WBC count at admission with adverse outcomes (mortality and bleeding) in patients undergoing coronary revascularization with cardiopulmonary bypass [11, 12]. However, in the case of cardiopulmonary bypass it is unclear whether high WBC count contributes to preexisting risk or to development of the systemic inflammatory response postoperatively or both.

The systemic inflammatory response is a complication in cardiopulmonary bypass patients that is caused by a combination of surgical stress and contact activation of blood component in the extracorporeal circuit [20, 21]. It is poorly defined [22] and the only formal definition is the Systemic Inflammatory Response Syndrome (SIRS), borrowed from the sepsis field [9]. According to the definition, SIRS exists when any two out of four criteria relating to abnormal temperature, heart rate, respiratory rate, or white cell counts exist. The upper threshold for abnormal white cell count according to the definition is 12,000 [9]. An evidence-based review of the inflammatory response indicated that all four SIRS criteria were rarely monitored in the setting of cardiopulmonary bypass [23] as they were felt to be too nonspecific [22] and if taken literally would apply to approximately 40% of all patients [24–26]. A more recent update on minimal reporting criteria by the Outcomes Consensus Panel singled out WBC count as the only criterion measured on its own as being relevant to the inflammatory status [10]. This recommendation was supported by other fields in which WBC count is recognized as a valid marker of inflammation [5, 6, 8, 27].

An alternative theory for the development of the systemic inflammatory response is that this is determined less by the extracorporeal circuit itself but rather by preexisting activation of white cells and endothelium [28] or by preoperative transfusion. Consistent with this theory is that high WBC count prior to coronary surgery utilizing cardiopulmonary bypass is linked with adverse outcomes including mortality and bleeding [11, 12]. Our present findings that high WBC count before-surgery is linked to an increased risk of 30-day readmission after discharge add further weight to this idea. We therefore conclude that WBC count measured prior to cardiac surgery may serve as a measure of the patient's inflammatory status and could aid in identifying and managing patients at heightened risk of readmission after discharge from cardiac surgery. This becomes especially relevant in an era when higher than expected readmission rates may attract financial penalties to hospitals.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors thank and acknowledge those allied health professionals that contributed to the development of the data collection form for readmissions and conducting the chart reviews including Cathy Ross, David Malenka, Kristine Chaisson, Rick Boss, Robert Helm, Susan Horton, Patricia Hofmaster, Helen Desaulniers, Benjamin Westbrook, Dennis Duquette, Kelly LeBlond, Cheryl Jones, Reed Quinn, and Patrick Magnus. The Northern New England Cardiovascular Disease Study Group is with Dartmouth-Hitchcock Medical Center, Clinical Research, Rubin Building 5th Floor, One Medical Center Drive, Lebanon, NH, USA. The study was funded in part by the Northern New England Cardiovascular Disease Study Group. Dr. Brown is supported by Grant no. K01HS018443 from the Agency for Healthcare Research and Quality on Patient Safety and Acute Kidney Injury.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 601424, 4 pages
http://dx.doi.org/10.1155/2013/601424

Review Article

Metabolic Acidosis Treatment as Part of a Strategy to Curb Inflammation

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Received 16 April 2013; Revised 22 May 2013; Accepted 22 May 2013

Academic Editor: Robert Clive Landis

Abnormalities in systemic acid-base balance may induce significant changes in the immune response, and they may play a significant role in the development or maintenance of immune dysfunction. Different forms of acidosis (metabolic and respiratory) and even different types of metabolic acidosis (hyperchloremic and lactic) may produce different effects on immune function. If alkalization has, or not, some effect on inflammation control is still a matter of speculation. Studies concerning these subjects are limited justifying this paper.

1. Introduction

Abnormalities in systemic acid-base balance may cause significant changes in the immune response. The clinical significance of these changes is not yet fully known, but its magnitude suggests that they may play a significant role in the development or maintenance of immune dysfunction. Thus, they represent attractive targets for curbing inflammation.

Metabolic acidosis is one of the most common abnormalities in patients suffering from serious diseases. There have numerous etiologies and treatment of the underlying disease is the basis of therapy. However, there is a growing evidence suggesting that acidosis itself has profound effects on the host, particularly in immune function. Given the critical importance of immune function for the outcome of the illness, there is an overriding interest in elucidating the effects of this condition.

In fact, recent evidence suggests that the different forms of acidosis (metabolic and respiratory) and even different types of metabolic acidosis (hyperchloremic and lactic) may produce different effects on immune function. The ways in which these effects are applied to the clinical conditions have not been determined. Therefore, since acidosis is an extremely common problem in intensive care units and that immune function is of vital importance, efforts to explain these relations are fully justified [1]. However, it is necessary to note that the publications linking acidosis with the inflammatory response are limited, and studies on the alkalosis are virtually nonexistent, justifying the current paper, at least as an open discussion (Figure 1).

2. Pathophysiology

The literature has reported in vitro experiments where researchers reduced intracellular pH (pHo) using different types of acids. Notably, different patterns of expression of mediator of inflammation occurred at different acids, despite the normalization of samples to the same pHo [1]. Kellum et al. [2] demonstrated that different degrees of hyperchloremic acidosis produce different effects on the release of inflammatory mediators. By using electrophoretic mobility shift, these researchers have measured the binding of NF-κB DNA after exposure to different concentrations of HCl. When compared to pHo 7.4, acidosis (7.0 pHo) significantly increased the activation of NF-κB induced by LPS,

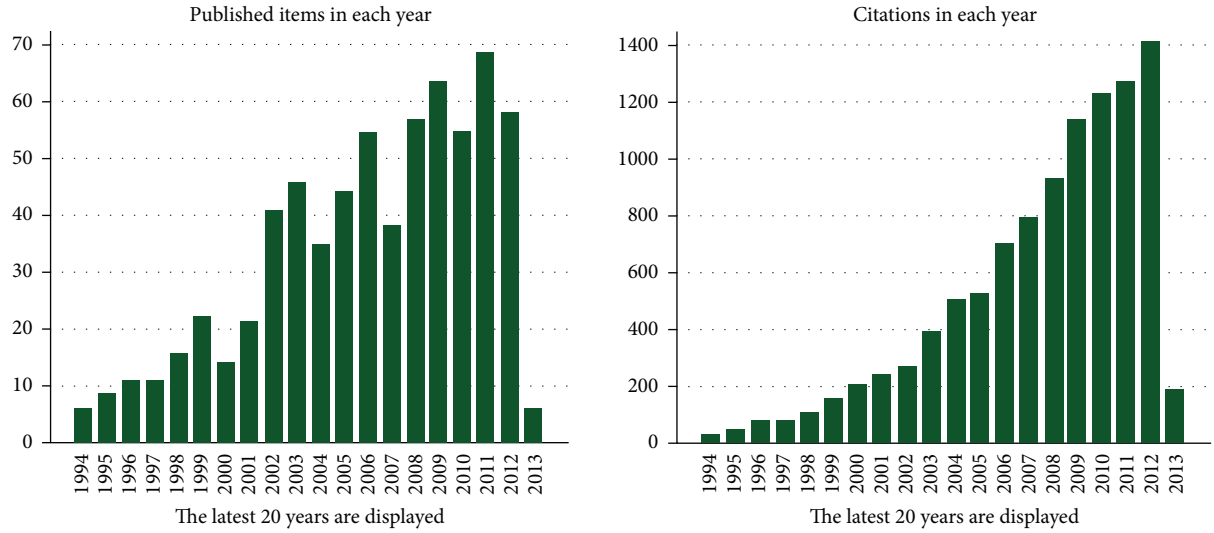

Figure 1: Metabolic acidosis and inflammation (Web of Science data).

while more severe acidosis (6.5 pHo) attenuated the activation of NF-κB, concluding that the degree of acidosis directly influences the immune function.

Unlike HCl, lactic acid has been studied in an even more limited way with even less convincing effects, and the mechanisms by which the HCl and lactate exert effects on innate immunity are unknown. Furthermore, the Kellum et al. investigation [2] evaluated the inflammatory response induced by LPS (*E. coli* toxin) associated with acidosis. The inflammatory response through quantification of inflammatory markers (NO, IL6, and IL-10 binding with DNA LPS), showed that HCl and lactic acid exhibit antagonistic responses, and an immune response increases with HCL and decreases with lactic acid. The basic conclusion is that different types of acids produced different effects on cellular immune function even when normalized to the same pH.

Evidently, the interrelationship between extracellular and intracellular pH on immune function cannot be ignored, especially in light of the numerous findings implicating a role for the Na/H exchanger prior to the activation of certain immune activities, suggesting that the Na/H exchanger is a *sine qua non* in generating a rapid intracellular alkalinization prior to the differential activation of certain immune activities [3]. However, a major criticism of the experimental studies is the strong acidification (pH 6.5–7.0), which shows more clearly the role of inflammation, and these levels of acidosis are rarely observed in the clinical setting. Moreover, it is difficult to differentiate between intracellular and extracellular acidosis, including possible "critical windows" and their relationship to inflammation.

3. Acid-Base Biomarkers and Inflammation

Farwell and Taylor conducted a survey about the relationship between serum acid-base status and inflammation. This study examined the relationship between serum anion gap, bicarbonate levels, and inflammatory biomarkers in healthy subjects. It was shown that a higher anion gap and a lower level of serum bicarbonate (despite being within the normal range) were associated with higher levels of several inflammatory biomarkers, including leukocyte count and levels of C-reactive protein. The cause of the observed relationship between higher serum anion gap and higher levels of inflammatory markers in this apparently healthy people is unknown. These data raise the possibility that the increased production of organic acids measured the chronic inflammatory disease, which increases the risk of coronary heart disease and cancer. More studies are necessary to determine the effectiveness of alkali supplementation on levels of inflammatory biomarkers [4].

Lactate clearance, a surrogate for the magnitude and duration of global tissue hypoxia, is used diagnostically, therapeutically, and prognostically. Nguyen and colleagues examined the association of early lactate clearance with selected inflammatory, coagulation, apoptosis response biomarkers, and organ dysfunction scores in severe sepsis and septic shock [5]. They carried out measurements of serum arterial lactate, and biomarkers (interleukin-1 receptor antagonist, interleukin-6, interleukin-8, interleukin-10, tumor necrosis factor-alpha, intercellular adhesion molecule-1, high mobility group box1, D-dimer, and caspase-3). In addition, organ dysfunction scores (Acute Physiology and Chronic Health Evaluation II, Simplified Acute Physiology Score II, Multiple Organ Dysfunction Score, and Sequential Organ Failure Assessment) were obtained in conjunction with a prospective, randomized study. Lactate clearance was defined as the percent change in lactate levels after six hours from a baseline measurement in the emergency department. The results of the statistical analysis showed that early lactate clearance as a surrogate for the resolution of global tissue hypoxia was significantly associated with decreased levels of biomarkers, improvement in organ dysfunction, and outcome in severe sepsis and septic shock. This well-designed study was selected because it illustrates the relationship between metabolic acidosis and the inflammation reaction.

4. Therapeutic Aspects

The various studies described above suggest that changes in systemic acid-base balance can also cause alterations in the immune response through several different ways. Thus, further investigation of these changes may lead to valuable therapeutic targets in treating some potentially serious diseases.

Acidosis and changes in intracellular and extracellular pH may influence endothelial function in different aspects. In large arteries, intracellular acidosis is associated with vasodilatation, whereas in small arteries, it leads to vasoconstriction. The pathogenic events of this response are not well known, but it was demonstrated that acidosis regulates not only the iNOS but also the eNOS [6]. Nitric oxide can contribute to the control of local blood flow during hypoxia/ischemia, which presents a close relationship with lactic acid production. This analysis suggests that the pH regulation may represent a potential therapeutic target in curbing inflammation. This new approach may attenuate endothelial dysfunction in diseases associated with acidosis [7].

Nowadays to consider what the best strategies to treat metabolic acidosis, considering the inflammatory response is an exercise in speculation and hypotheses. For example, there are advantages to seek alternative ways to treat intracellular acidosis, or just to know that it comes concomitant to the extracellular treatment? If have advantages, "resurrect" the TRIS buffer clinical use would be considered since this buffer is theoretically more effective to treat the intracellular acidosis, than ever questioned bicarbonate?

The "folk" thought that the patient died of multiple organ failure with hemogasometry normal is particularly metaphorical and capable of deep reflection. The correction of metabolic acidosis as an isolated marker needs to be abandoned and considered being an essential part of the systemic inflammatory response. Thus, administration of potent and selective NHE1 inhibitors afford protection from the whole body ischemia-reperfusion injury by attenuating myocardial dysfunction and improving organ blood flow and systemic oxygen delivery, resulting in reduced proinflammatory response [8].

5. Conclusion

Most often, metabolic acidosis is present in acute systemic inflammatory response in which the control of acid-base balance is part of the treatment protocol. Thus, evaluation of the role of metabolic acidosis is mandatory. One of main concerns about this paper is the difficulty to establish "what the chicken is and what the egg is." In many cases, acute acidosis is secondary to, for example, circulatory shock, and one could wonder whether, under those conditions, the circulatory shock causes the inflammatory response or the acidosis related to the shock. The same reasoning can be followed in patients who develop respiratory acidosis due to ARDS or COPD, as the lung disease by itself will induce an inflammatory response. Perhaps the most unequivocal data providing evidence for an impairment of the immune response appear from the clinical studies of the organic acidosis and ketoacidosis. In general, the clinical acidemias are accompanied by immunodeficiency, including a reduction in white cell numbers, gamma globulins, mitogenic responses, a diminution of the inflammatory response, and delayed phagocytosis. In many cases, the immunodeficiency is reversed on the correction of the acidosis. Despite the valuable research carried out to date, a lack in the appreciation of extracellular acid-base effects on a wide range of other immune activities exists [3]. Therefore, the situations in which acidosis remains steadily, as chronic renal failure, would be more suitable for the evaluation of the treatment to curb the spread of the inflammatory process.

It should be emphasized that the metabolic acidosis is common in critically ill patients and its presence can have a detrimental effect on clinical outcome. The administration of base, a common therapeutic maneuver, does not appreciably improve clinical outcome, even when acidosis is improved [9]. Is it better to consider "body acid-base imbalance" than "blood acid-base imbalance"?

Metabolic Acidosis and Inflammatory Response Key Points

(i) Metabolic acidosis is one of the most common abnormalities in patients suffering from serious diseases, and there is a growing evidence suggesting that acidosis itself has profound effects on the host, particularly in immune function.

(ii) Recent evidence suggests that the different forms of acidosis (metabolic and respiratory) and even different types of metabolic acidosis (hyperchloremic and lactic) may produce different effects on immune function.

(iii) Publications linking acidosis with the inflammatory response are limited, and a major criticism of the experimental studies is the strong acidification (pH 6.5–7.0), which shows more clearly the role of inflammation, and these levels of acidosis are rarely observed in the clinical setting.

(iv) Anion gap, bicarbonate, and lactate are possible biomarkers of the inflammation response.

(v) Perhaps the most unequivocal data providing evidence of the immune response impairment emerge from the clinical studies of the organic acidosis and ketoacidosis. In general, the clinical acidemias are accompanied by immunodeficiency, including a reduction in white cell numbers, gamma globulins, and mitogenic responses, a diminution of the inflammatory response.

(vi) Nowadays, to consider what the best strategies to treat metabolic acidosis, considering the inflammatory response is an exercise in speculation and hypotheses.

(vii) The administration of selective inhibitors of NHE1 minimizes the degree of cellular injury and improves survival.

(viii) The correction of metabolic acidosis as an isolated marker needs to be abandoned and considered as being an essential part of the systemic inflammatory response. Is it better to consider "body acid-base-imbalance" than "blood acid-base-imbalance"?

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 183061, 5 pages
http://dx.doi.org/10.1155/2013/183061

Review Article

Curbing Inflammation in the Ischemic Heart Disease

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Received 13 February 2013; Accepted 14 May 2013

Academic Editor: Robert Clive Landis

A modern concept considers acute coronary syndrome as an autoinflammatory disorder. From the onset to the healing stage, an endless inflammation has been presented with complex, multiple cross-talk mechanisms at the molecular, cellular, and organ levels. Inflammatory response following acute myocardial infarction has been well documented since the 1940s and 1950s, including increased erythrocyte sedimentation rate, the C-reactive protein analysis, and the determination of serum complement. It is surprising to note, based on a wide literature overview including the following 30 years (decades of 1960, 1970, and 1980), that the inflammatory acute myocardium infarction lost its focus, virtually disappearing from the literature reports. The reversal of this historical process occurs in the 1990s with the explosion of studies involving cytokines. Considering the importance of inflammation in the pathophysiology of ischemic heart disease, the aim of this paper is to present a conceptual overview in order to explore the possibility of curbing this inflammatory process.

1. Introduction

Inflammatory response following acute myocardial infarction (AMI) has been documented since the 1940s and 1950s, including increased erythrocyte sedimentation rate (ESR), the C-reactive protein analysis (CRP), and the determination of serum complement (C′). Boltax and Fischel (1956) using serial assay of the ESR, C′, and CRP in sixty-one AMI episodes observed that such tests were positive in over 90% of patients by the third day from the onset of the disease [1].

In 1943, Lofstrom reported that patients with myocardial infarction also presented the "non-specific capsular swelling in pneumococci," later associated with the presence of the "C-reactive protein" [2]. Since then, a number of studies have confirmed the occurrence of CRP in myocardial infarction and other noninfectious inflammatory conditions [3, 4]. Surprisingly, an extensive literature overview including publications from 1960s to the 1980s revealed that the role of the inflammation in the AMI lost relevance, virtually disappearing from the literature reports. The reversal of this historical process occurred in the 1990s with the upsurge of investigations involving cytokines (Figure 1).

Therefore, considering the importance of inflammation in the pathophysiology of ischemic heart disease (IHD), the aim of this review is to present an overview of concepts in order to explore the possibilities for curbing the inflammatory process associated with myocardial infarction.

2. Inflammation and Ischemic Heart Disease

Nowadays acute coronary syndrome (ACS) has been considered an autoinflammatory disorder comprising the molecular, cellular, and organ multiple cross-talk mechanisms. Even though, early reperfusion, either by thrombolysis or percutaneous coronary intervention, provides excellent clinical benefits in patients with ACS, the ischemia/reperfusion injury may somewhat offset those positive advantages. Although being potentially protective, inflammation has been associated with potentially detrimental conditions such as activation of leukocytes, endothelial cells, vascular smooth muscle cells, platelets, and oxidative stress [5].

Therefore, the inflammation in response of ischemia and necrosis of cardiac tissue has a crucial role not only in tissue repair but also in the prognosis of patients. Biasucci

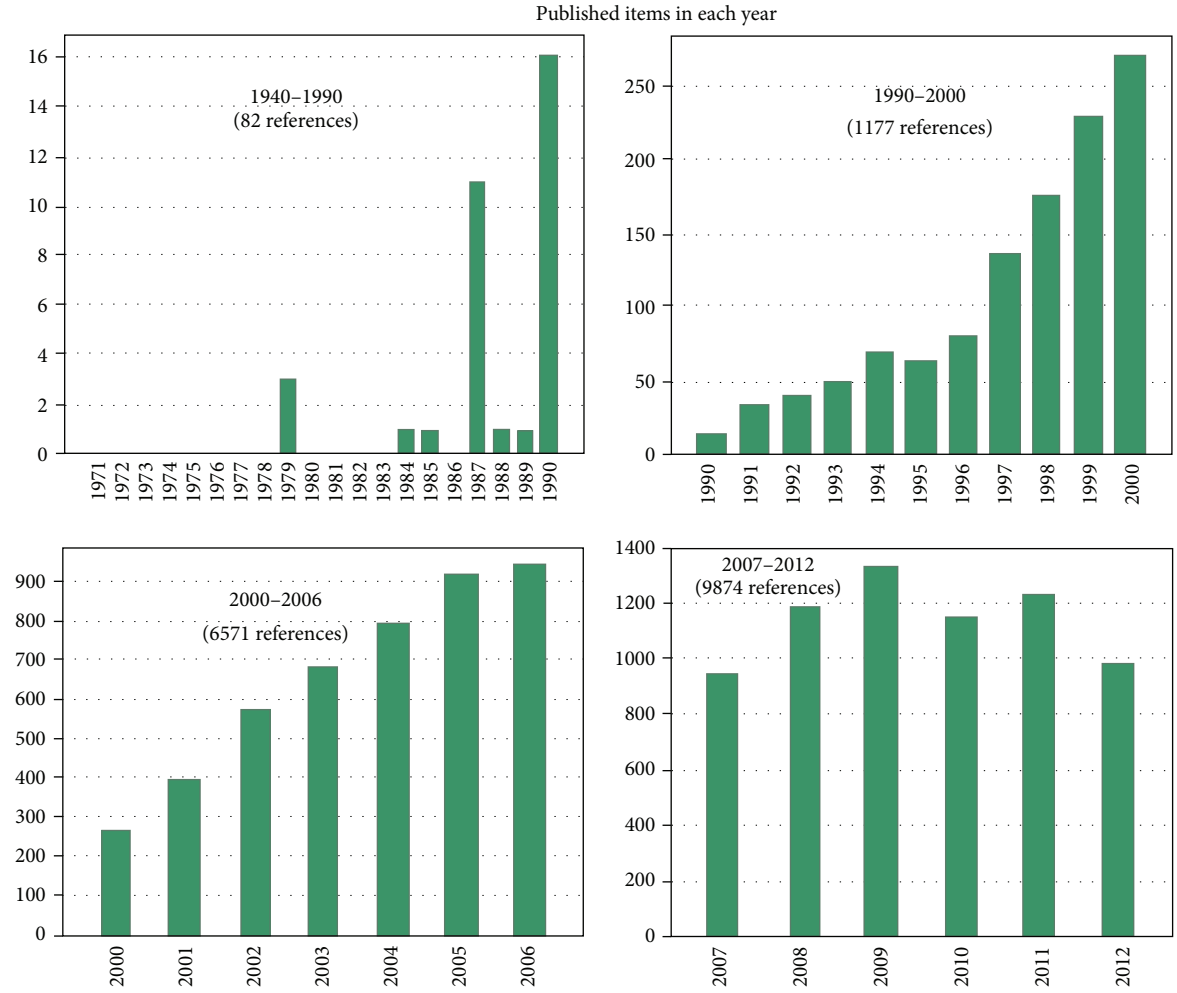

Figure 1: Web of Science timespan references (1940–2012).

and colleagues (2000) summarized the current concepts of the inflammatory reaction associated with coronary artery disease (CAD). In patients with unstable angina, coronary atherosclerotic plaques are characterized by the presence of macrophages, and to a lesser extent, T-lymphocytes, at the immediate site of either plaque rupture or superficial erosion. Moreover, the rupture-related inflammatory cells are activated, indicating ongoing inflammation at the site of plaque disruption. These observations corroborate the results of clinical studies demonstrating activated circulating neutrophils, lymphocytes, and monocytes, increased concentrations of proinflammatory cytokines, such as interleukin (IL) 1 and 6, and acute phase reactants in patients with unstable angina and myocardial infarction. High levels of C-reactive protein have been associated with an increased risk of in-hospital and later new coronary events in patients with unstable angina, as well as with increased long term risk of death and myocardial infarction in apparently normal subjects. Hence, the cumulative evidences suggest that inflammation may cause local endothelial activation and plaque fissure resulting in unstable angina and myocardial infarction. Although no information is available about why, when, and where exactly the inflammatory process begins, these concepts stimulate researches that may lead to a different approach to the patients with acute coronary syndromes [6].

Two different inflammatory processes take place in patients experiencing AMI. One is in the coronary arterial inflammation that results in AMI and the other occurs in the myocardial and leads to ventricular remodeling. These processes are positively and negatively regulated by Th1 and Th2 lymphocytes, respectively. In an investigation to clarify whether the T-helper (Th)1/Th2 imbalance is involved only in the coronary arteries inflammation or also in the myocardial inflammation and also to explore the importance of the imbalance of Th1/Th2 in the AMI, Cheng and colleagues (2005) observed that IFN-gamma-producing T cells were significantly increased in patients with AMI and unstable angina within 24 hours after the onset of symptoms. They also observed that the high ratio of IFN-gamma-producing T cells had normalized 1 week after the recovering of an unstable angina episode but was still observable 1 week and even 1 month after the AMI. The upregulation of Th1 cell function is compatible with a diseased heart function. There was no significant difference in the frequencies of

IL-4-producing T cells 1 week, 2 weeks, and 1 month after AMI. IFN-gamma mRNA increased in the myocardium of rats, but there was no significant change in global Th cell functions. The conclusions were (1) Th1/Th2 functional imbalance exists in both coronary arterial inflammation and myocardial inflammation processes and (2) the upregulation of Th1 cell functions may participate in the immune-mediated ventricular remodeling after AMI [7].

3. Acute Myocardial Infarction and Systemic Inflammatory Response

Cardiogenic shock is a devastating consequence of AMI associated with extremely high mortality. The treatment focuses on improving myocardial perfusion/reperfusion and hemodynamic support. Therefore, the main approach is an emergency angiography followed by coronary revascularization by percutaneous intervention or coronary artery bypass grafting. Circulatory support using diastolic intra-aortic balloon pump is frequently used in association with the pharmacological support with vasoactive and inotropic drugs, even though their benefit on survival has not been shown [8].

Recent lines of evidence suggest that systemic inflammatory response, including iNOS upregulation, complement activation, and the cascade of inflammatory cytokines, have a role in the development of cardiogenic shock. Therefore, new strategies to restrain the inflammatory process, including the use of C5 and NOS inhibitors, would be combined to the traditional strategies to treat cardiogenic shock.

Since the systemic inflammatory response (SIRS), complement activation, release of inflammatory cytokines, expression of inducible NO synthase (iNOS), endothelial activation, and inappropriate vasodilatation play a critical role in the genesis as well as in the evolution of the cardiogenic shock, new interpretations and therapeutic strategies have been evolved to deal with this ominous consequence of the AMI, as exposed by Reynolds and Hochman (2008) [9].

The tilarginine, an LN-monomethyl arginine (L-NMMA) or N(G)-monomethyl-L- arginine HCL, is a nonselective inhibitor of nitric oxide synthase (NOS), which has been studied for treating septic shock and cardiogenic shock complicating myocardial infarction. There is evidence that overproduction of nitric oxide (NO) may contribute to the pathogenesis of cardiogenic shock after myocardial infarction, which is similar to the observed in septic shock. The results of investigations using NOS inhibition in those two disorders have proved disappointing. However, the use of an inducible NOS inhibitor for reducing the pathological effects of excessive NO production might be useful [10, 11].

However, the results of experimental researches in animals as well as in humans have been promising. However, investigations in humans (TRIUMPH) with a larger sample whose objective was to assess tilarginine have recently been terminated due to the lack of efficacy and the tendency to increased mortality. The unfavorable evidence of the iNOS inhibition in cardiogenic shock resulted in considerable challenging: "The tragedy of TRIUMPH inhibition of nitric oxide synthesis: where do we go from here" [12, 13].

Methylene blue (MB), a guanylate cyclase inhibitor, can abolish the relaxation of vascular smooth muscle cyclic GMP-dependent without interfering with the NO synthesis and tissue necrosis associated with the use of NOS inhibitors. Therefore, MB may be a therapeutic option, untested, for vasoplegia associated with cardiogenic shock [14, 15]

4. Biomarkers

Since myocardial infarction onset is usually easily timed, it is possible to evaluate the effectiveness of biomarkers in the course of the AMI [1]. Therefore, there have been line of evidence suggesting that new biomarkers combined with cardiospecific troponin, CPR and ERS, may increase the sensitivity of diagnosing acute coronary syndrome [16].

Atherosclerosis is an inflammatory disease, and increased blood levels of inflammatory biomarkers have been observed in acute coronary syndromes. In addition, high expression of inflammatory markers is associated with a worse CAD prognosis. Thus, the most frequent biomarkers used in humans and animal investigations are (1) plasma levels of cytokines IL-6, IL-8, and TNF-α; (2) membrane expression of Toll-like receptors 2 and 4; (3) CD11b, CD62L, and CD14 on monocytes and granulocytes as markers of inflammation [17].

Elevated CRP levels have been associated with serious adverse cardiac events including death. However, the causal association of CRP with atherogenesis is less clear, and there are data suggesting that it is a bystander rather than a true risk factor. Importantly, CRP levels decrease in response to anti-inflammatory agents, making it useful for monitoring the efficacy of novel anti-inflammatory drugs [18]. The ESR and CPR analyses are the oldest markers of AMI and are still useful on the clinical practice.

5. Curbing Inflammation

According to Klingenberg and Luscher (2012), there are several promising anti-inflammatory drugs that have been tested, and four aspects appear to be paramount for interpreting the results of future trials. First, an anti-inflammatory agent should interfere with inflammatory pathways known to be crucially involved in the pathogenesis of atherosclerosis, but unlike statins such anti-inflammatory agent should attenuate inflammation *per se* and not interfere with lipid levels or other risk factors. Second, a biomarker which reflects the activity of the inflammatory pathway would be required for monitoring the treatment. Third, appropriate identification of patients likely to benefit from this treatment is essential. Either individuals at high risk for cardiovascular events identified by traditional risk scores or patients at high risk for recurrent events after AMI may be considered proper candidates. Fourth, choosing an adequate time point within the natural course of atherosclerosis and the duration of therapy are vital considerations. Obviously, an anti-inflammatory therapy would only provide real clinical benefit

Table 1: Ischemic heart disease and inflammation—key physiopathology concepts.

(i) A modern concept considers ACS as an autoinflammatory disorder.
(ii) Inflammatory response following AMI has been well documented since the 1940s and 1950s.
(iii) It is surprising to note, based on extensive literature overview including the following 30 years (decades of 1960, 1970, and 1980), that the inflammatory AMI lost its focus, virtually disappearing from the literature reports.
(iv) There are two different inflammatory processes in patients with AMI: the coronary arterial inflammation that leads to the pathogenesis of AMI, followed by myocardial inflammation that leads to ventricular remodeling.
(v) Systemic inflammatory response (SIRS), complement activation, release of inflammatory cytokines, iNOS expression, and vasodilatation cannot only play a pivotal role in the genesis and evolution of shock.
(vi) The most frequent biomarkers used in humans and experimental protocols are (1) plasma levels of cytokines IL-6, IL-8, and TNF-α; (2) membrane expression of Toll-like receptor; (3) CD11b, CD62L, and CD14 on monocytes and granulocytes as markers of inflammation.
(vii) Curiously, increased erythrocyte sedimentation rate (ESR) and the C-reactive protein analysis (CRP) are the oldest markers of AMI and still are the most useful on the clinical practice.

Table 2: Curbing inflammation in ischemic heart disease—key points.

(i) An anti-inflammatory therapy would provide real clinical value if an incremental benefit above and beyond existing therapies in a cost-efficient approach could be provided.
(ii) A potential new therapeutic target of ACS includes at least four anti-inflammatory treatment options: (1) nonspecific anti-inflammatory drugs; (2) specific antagonists of key cytokines; (3) immunomodulatory therapies; (4) immunization as promising therapeutic modality against atherosclerosis.
(iii) There is an early inflammatory response (innate inflammation) that would be a protective reaction in the acute phase of MI. Over time, persisting inflammatory response should be curbed.
(iv) The onset of AMI is determined with a certain safety margin. Thus, based on the concepts of ischemic myocardial protection emanating from the 1970s, it would be inappropriate "curbing" inflammation within 6 hours.
(v) General inhibition of the innate immune system is associated with adverse outcome after the challenge being to inhibit those parts of the innate immune system that cause injury, without affecting the myocardial infarct healing.
(vi) Would the sense of genetic predisposition, based on sensitive biomarkers, be an initial step to get strategies for AMI curbing inflammation?
(vii) It is well known that the inflammation occurs in the coronary artery wall, in the atherosclerotic plaque, and the myocardium. Would these alterations be considered individually or as a part of a single process of inflammation?
(viii) Would regular medications (ACE inhibitors, statins, aspirin, nitrates, and beta-blockers) be no longer functioning as curbing the AMI inflammatory process?

if its effectiveness is beyond that of existing usual therapies and cost effective [18].

A number of experimental and clinical investigations have highlighted the key role of inflammation in all phases of atherosclerosis, from fatty streaks to disrupted plaques. Higher levels of inflammatory markers have been associated with poor outcome despite the optimal treatment, including myocardial revascularization. In a thorough review Bona and colleagues focused on inflammation as a potential new therapeutic target of ACS appraising four anti-inflammatory treatments: (1) nonspecific anti-inflammatory drugs; (2) specific antagonists of key cytokines; (3) immunomodulatory therapies; (4) immunization as promising therapy against atherosclerosis [19]. Klingenberg and Luscher (2012) have published another worthy review [18], and both reviews are essential for those interested in potential therapeutic strategies for "curbing inflammation."

The early inflammatory process, the innate inflammation, would be a protective reaction in the acute phase of myocardial infarction. However, the overtime inflammatory response should be curbed. Therefore, based on the concepts of ischemic myocardial protection established in the 1970s, it would be inappropriate to curb inflammation within 6 hours after the onset of AMI. However, according to Timmers and colleagues (2012) translation of therapeutic anti-inflammatory strategies to reduce myocardial ischemia/reperfusion injury into clinical practice appears to be a challenging task since general inhibition of the innate immune system is associated with adverse outcomes after myocardial infarction. The challenge is to inhibit those parts of the innate immune system that cause injury without affecting the myocardial infarct healing. The current body of knowledge is limited to understand the spatial and temporal functions of endogenous ligands and their receptors, inflammatory cells, and inflammatory mediators with pleiotropic and synergistic or antagonistic effects in myocardial ischemia/reperfusion injury [20].

The natural history demonstrated that early reperfusion (thrombolysis, PTCA, and surgery) has a positive impact on the AMI evolution, resulting in a significant reduction of cardiogenic shock, ventricular aneurysms, and death. Thus, this presents a further question: should all patients undergo anti-inflammatory treatment or only those that are experiencing elevated levels of biomarkers, especially CRP, ESR, and

complement? In addition, based on the variety of individual clinical evolution after AMI (cardiogenic shock, progression to dilated cardiomyopathy, ventricular aneurysms, and SIRS), the involvement of genetic factors is clear. Thus, ascertain the genetic predisposition in conjunction with the presence of biomarkers of inflammation should be an initial step for curbing inflammation associated with AMI.

Finally, two hypothetical questions have to be addressed. It is well known that inflammation occurs in the wall of the coronary arteries, atherosclerotic plaque, and myocardium, raising the question if these processes should be considered individually or as part of a unique process of inflammation. In addition, one should consider if conventional medications (ACE inhibitors, statins, aspirin, nitrates, and beta-blockers) would be no longer functioning as curbing the inflammatory process associated with AMI?

Table 1 summarizes the physiopathological "key points", and "key points" for curbing inflammation in ischemic heart disease are summarized in Table 2.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 578480, 16 pages
http://dx.doi.org/10.1155/2013/578480

Review Article

Blocking Neurogenic Inflammation for the Treatment of Acute Disorders of the Central Nervous System

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Received 6 February 2013; Accepted 8 May 2013

Academic Editor: Christopher D. Buckley

Classical inflammation is a well-characterized secondary response to many acute disorders of the central nervous system. However, in recent years, the role of neurogenic inflammation in the pathogenesis of neurological diseases has gained increasing attention, with a particular focus on its effects on modulation of the blood-brain barrier BBB. The neuropeptide substance P has been shown to increase blood-brain barrier permeability following acute injury to the brain and is associated with marked cerebral edema. Its release has also been shown to modulate classical inflammation. Accordingly, blocking substance P NK1 receptors may provide a novel alternative treatment to ameliorate the deleterious effects of neurogenic inflammation in the central nervous system. The purpose of this paper is to provide an overview of the role of substance P and neurogenic inflammation in acute injury to the central nervous system following traumatic brain injury, spinal cord injury, stroke, and meningitis.

1. Introduction

Acute disorders of the central nervous system (CNS), including traumatic brain injury (TBI), spinal cord injury (SCI), stroke, and meningitis, account for a significant disease burden worldwide, with CNS injury being the leading cause of death after trauma [1]. These acute neurological conditions affect individuals of all ages and both sexes alike resulting in significant morbidity and mortality. Despite the prevalence of these conditions, current treatments remain limited and largely inadequate. New therapies are urgently required in order to reduce the death and disability associated with these conditions. One feature which is central to each of these conditions is disruption to the blood-brain barrier (BBB)/blood-spinal cord barrier (BSCB) and subsequent development of vasogenic edema. As such, targeting this aspect of the injury cascade is likely to produce beneficial outcomes in each of these conditions. Recent reports on the role of the neuropeptide substance P (SP) and neurogenic inflammation in BBB dysfunction and genesis of cerebral edema following acute brain injury suggest that this pathway provides a novel target for therapeutic intervention. The current paper will provide an overview of the BBB and vasogenic edema, followed by a discussion of the role of SP and neurogenic inflammation in CNS injury.

2. Blood-Brain Barrier/Blood-Spinal Cord Barrier

The BBB is a highly selective barrier that serves to protect the fragile brain microenvironment. It is the interface between the blood and the brain, separating the brain parenchyma from the blood within cerebral capillaries, and involves the interactions between endothelial cells, astrocytes, pericytes, and the capillary basement membrane. Within the spinal cord, the blood-spinal cord barrier (BSCB) is similar in function to the BBB [2] and serves to protect the spinal cord by modulating the entry of blood-borne substances. The fundamental structures of the BBB and BSCB are the same although there are some specific differences in the BSCB including glycogen deposits, decreased P-glycoprotein transporters, and decreased expression of tight junctional protein expression [3].

The main function of these barriers is to facilitate a constant supply of nutrients, preserve ion homeostasis within the brain/spinal cord microenvironment, and protect against

noxious chemicals, variations in blood composition, and the breakdown of concentration gradients. The gate function of the BBB and BSCB is afforded by tight and adherens junctions, comprised of a complex network of transmembrane and cytosolic proteins [4, 5]. Specifically, claudins, occludins, junctional adhesion molecules (JAMs), and zona occludens (ZOs) are the proteins that make up this network. Tight junctions are located on the most apical region of the cleft between cerebral capillary endothelial cells and form a seal to prevent substances from passing between them [6]. Claudins, predominately caludin-5, are involved in the primary makeup or backbone of tight junctions, forming dimers which interact with opposing claudin molecules to form the primary seal of the tight junction [6, 7]. JAM has a single transmembrane segment, which initiates cell-to-cell attachment and is able to mediate permeability through this avenue [7]. Occludin has four transmembrane segments and is present in higher concentrations in endothelial cells of the BBB than in those in systemic capillary endothelial cells. It induces high membrane resistance, which is indicative of low ion permeability [7, 8]. Occludin interacts with the cytoskeleton of BBB/BSCB endothelial cells through ZO1, ZO2, and ZO3 molecules [6, 7]. A further obstacle to prevent the entry of unwanted substances into the brain is provided by the basement membrane of the BBB, which is made up of proteins found within the extracellular matrix including collagens, vitronectin, fibronectin, tenascin, and proteoglycans [9]. These components provide stability to the structure of the blood vessels and a surface upon which cerebral capillary endothelial cells can rest.

Astrocytes are central to the structure and function of the BBB/BSCB. Their end feet surround 99% of BBB endothelial cells and act to support and enhance the tight junctions between them [7, 10]. Furthermore, astrocytes mediate the connection between neurones and endothelial cells [11], and the gap junctions between astrocytes allow for quick transfer of substances and information [12]. They become activated in response to pathological stimuli, which results in the hypertrophy of the astrocytic processes and overexpression of intermediate filaments, namely, glial fibrillary acidic protein [12].

Pericytes have a stellate appearance and cytoplasmic processes and act as support cells that play an important role in the BBB/BSCB. They cover 22–32% of the capillary cell surfaces [13], and the gap junctions between pericytes and cerebral capillary endothelial cells allow communication to occur [7]. The main function of pericytes is thought to be blood flow regulation, particularly in the precapillary arterioles that supply the brain with blood [14]. The structure of pericytes makes them ideal for this function, as they are contractile and express the smooth muscle actin isoform [13]. Collagen type IV glycosaminoglycans and laminin are also synthesised in pericytes to be used in formation of the basement membrane [13]. They have the ability to regulate endothelial cell proliferation, survival, migration, and differentiation [7].

3. Edema

Of the secondary injury factors that occur in the setting of CNS injury, edema within the brain or spinal cord is of particular concern given its association with increased mortality and morbidity [15, 16]. Edema is defined as the abnormal accumulation of fluid within the CNS tissue. Klatzo [17] was the first to classify edema into two broad categories based upon the integrity of the BBB: cytotoxic and vasogenic edema. Cytotoxic edema is an intracellular edema that occurs as a result of cellular injury. It is characterized by a shift of water from the extracellular compartment to the intracellular compartment, accompanied by shrinkage of the extracellular space. Cytotoxic edema occurs independently of alterations in the BBB/BSCB and appears to be more prominent in the grey matter [18]. Failure of the Na^+/K^+ ATPase in regions of energy failure and subsequent loss of ion homeostasis, leading to influx of water into cells, is central to the development of cytotoxic edema [19, 20]. Conversely, vasogenic edema has been shown to be more prevalent in the white matter [18] and involves the escape of proteins from the vasculature in the setting of BBB/BSCB disruption and injury to cerebral blood vessels. Protein accumulation in the brain/spinal cord extracellular space causes an osmotic increase at the site of injury and the subsequent movement of water down its osmotic gradient. There is a strong correlation between extravasation of proteins into the extracellular space and the development of vasogenic edema [21, 22].

The temporal profile of edema pathogenesis after injury varies greatly with injury type and severity [23] and has been extensively studied in order to characterize the period in which anti-inflammatory pharmacological interventions may be effective. In a mouse model of cerebral contusion, permeability of the BBB to large proteins was resolved by approximately 5 hours following injury, whereas smaller molecules of 10 kDa were still able to pass through the BBB for up to 4 days [24]. Similarly, the BSCB may be disrupted for several days following traumatic SCI [25, 26]. Furthermore, in ischemic stroke, it has been shown that edema continues to develop for up to 7 days, with the initial cytotoxic edema being followed by vasogenic edema [27]. Thus, there is substantial opportunity for amelioration of barrier dysfunction and subsequent cerebral edema through manipulation of mediators of BBB/BSCB permeability. Further studies are required to elucidate the exact mechanisms of barrier disruption and subsequent edema pathogenesis to develop targeted therapeutic agents.

The development of edema is associated with significant mortality and morbidity in the setting of CNS injury. Such outcomes are related to the ability of vasogenic edema to lead to an increase in pressure within the cranium or spinal canal. Given that the skull is rigid structure, any increase in the intracranial contents (blood, brain, and cerebral spinal fluid) must be compensated by a decrease in the volume of the other components. The same is true within the spinal column. Within both the brain and the spinal cord, there is limited capacity for compensation through reductions in blood or cerebrospinal fluid volume to accommodate for an increase in the intracranial volume. This compensation is responsible for the initial plateau in the intracranial pressure/volume curve, which becomes exponential once compensatory mechanisms are exhausted [28]. When such compensatory mechanisms fail, profound increases in intracranial pressure (ICP) or

intrathecal pressure (ITP) may result. The sequelae of elevated ICP/ITP include reduced blood flow to CNS tissue, ischemia and infarct extension, deformation and herniation of the brain and spinal cord tissue, and in severe cases, death [18, 29–31].

With the mortality of malignant cerebral edema approaching 80% [18], the reduction of cerebral edema and its associated rise in ICP is now widely recognised as an important clinical management target. Current treatments seek to reduce brain swelling and ICP though administration of hyperosmotic agents and barbiturates, induction of hyperventilation or hypothermia, and surgical interventions such as cerebrospinal fluid (CSF) drainage, or in severe cases, decompressive craniectomy [23, 30, 32, 33]. In the case of hemorrhage, evacuation of space occupying lesions like hematomas may be warranted [34]. Clinical signs of edema have been linked with poor functional outcome following SCI [16]. The use of steroids in an attempt to minimize SCI-induced edema and inflammation is common, despite the controversy surrounding their effectiveness and safety [35]. Decompressive surgery is also a current standard treatment following SCI [36].

With respect to patient morbidity and mortality, current clinical treatment regimens for acute disorders of the CNS have proven somewhat ineffective, mainly because they do not address the specific mechanisms that are associated with the genesis of edema in cerebral ischemia. Recent studies have identified substance P (SP) release as a feature of acute CNS injury and have delineated a critical role for SP in increased BBB permeability and the development of vasogenic edema.

4. Neurogenic Inflammation

Neurogenic inflammation is a neurally elicited, local inflammatory response characterized by vasodilation, increased vascular permeability, mast cell degranulation, and the release of neuropeptides including SP and calcitonin gene-related peptide (CGRP) [37]. In addition, there are also tissue-specific responses including smooth muscle contraction/relaxation in the bladder and bronchoconstriction in the airways, amongst others [38]. Neurogenic inflammation has been demonstrated in tissue receiving trigeminal innervation and may be stimulated by many agents including prostanoids, leukotrienes, histamine, and serotonin, as well as by changes in the extracellular environment such as decreased pH, increased osmolarity, heat, inflammatory conditions, and tissue (mechanical) injury [39, 40]. The changes in blood vessel size and permeability that occur with neurogenic inflammation lead to edema formation within the tissue [21, 22]. Perhaps the most important factor in this response is SP, having been identified as the most potent initiator of neurogenic inflammation [41, 42].

Neurogenic inflammatory mediators such as SP and CGRP and their respective receptors are found in abundance in both the rodent and human CNSs, and whilst neurogenic inflammation and classical inflammation are both inflammatory processes, neurogenic inflammation in the brain differs from classical inflammation in that neurogenic inflammation is neurally elicited and results in an increased

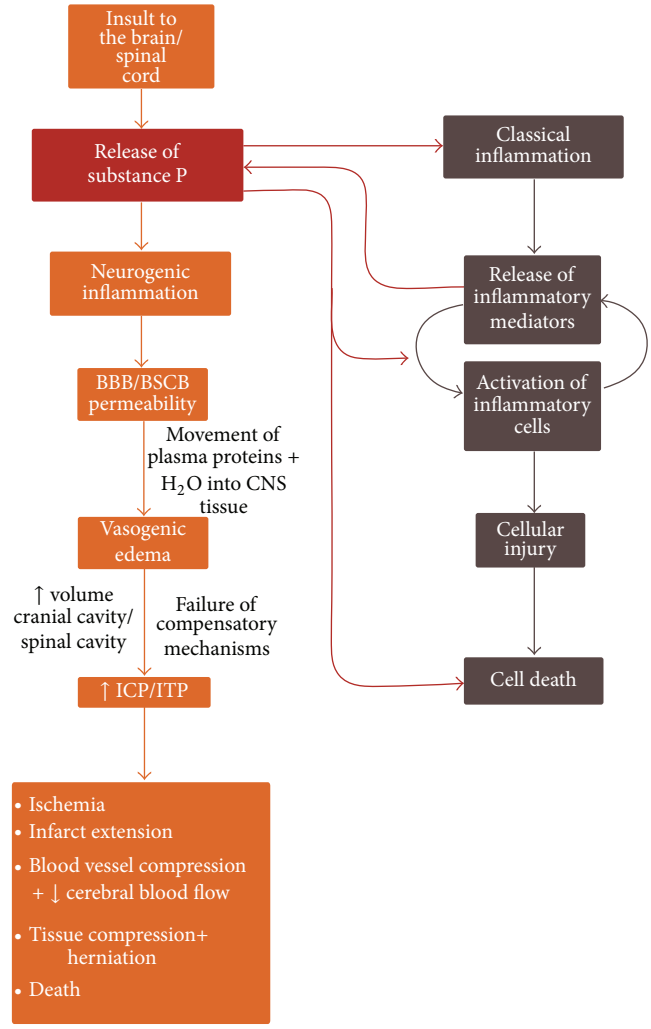

Figure 1: Acute CNS injury leads to the initiation of both neurogenic inflammation and classical inflammation.

permeability of the BBB through the release of neuropeptides. In contrast, classical inflammation involves the accumulation and proliferation of microglia, perivascular macrophages, and other inflammatory cells (Figure 1) [43, 44]. These cells subsequently release classical inflammatory mediators like bradykinin, which drive vascular changes [45]. Nevertheless, there is an interaction between the two processes as many of the factors within each cascade may initiate or potentiate the other. For example, the classical inflammatory mediator bradykinin causes release of the neurogenic inflammatory mediator SP, which in turn is well known to cause mast cell degranulation along with bradykinin and nitric oxide release by endothelial cells and thus potentiation of classical inflammation (Figure 1). Inflammation in the brain may play many roles, including the maintenance of tissue homeostasis, although when these processes are unable to be controlled, tissue damage occurs. Thus, this paper focuses on the pharmacological blockade of neurogenic inflammation for the treatment of acute disorders of the CNS.

There are multiple pathways by which neurogenic inflammation may be initiated. It is well documented, using both

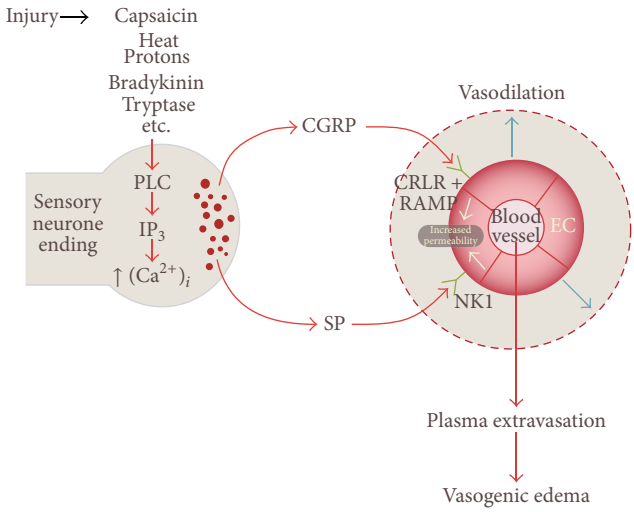

Figure 2: Neurogenic inflammatory initiation of vasogenic edema. PLC—phospholipase C, IP_3—inositol triphosphate, $(Ca^{2+})_i$—intracellular calcium ions, CGRP—calcitonin gene-related peptide, SP—substance P, CRLR—calcitonin receptor-like receptor, RAMP—receptor activity modifying protein, NK1—NK1 receptor, EC—endothelial cell.

animal models and isolated neurons in vitro, that capsaicin, heat, protons, bradykinin, and tryptase are upstream regulators of the intracellular calcium influx, which results in inflammatory neuropeptide release [46–48]. In contrast, it is thought that prostaglandins E_2 and I_2, cytokines, interleukin-1, interleukin-6, and tumor necrosis factor do not cause neurotransmitter release themselves, but rather excite sensory neurons and thus lower the threshold for firing and cause augmented release of neuropeptides [48, 49].

While neurogenic inflammation has been extensively studied and well documented in peripheral tissues [50, 51], until recently the concept of neurogenic inflammation within the CNS has remained largely unexplored. Given the capacity for neurogenic inflammation to influence vascular permeability and lead to the genesis of edema (Figure 2), it has now been widely investigated for its potential to influence BBB permeability and vasogenic edema within the brain and spinal cord under varying pathological conditions.

4.1. Capsaicin. Capsaicin activates transient receptor potential vanilloid-1 (TRPV1) channels on polymodal nociceptive fibers, thus, resulting in the release of neurogenic inflammatory mediators and subsequent vasodilation and increased vascular permeability [52, 53]. Under experimental conditions, capsaicin is commonly used to cause release and/or depletion of neuropeptides [54]. Thus, capsaicin initially elicits a neurogenic inflammatory response, followed by a refractory period in which there is no response to factors that would ordinarily induce neurogenic inflammation. At high doses in young animals, capsaicin may cause permanent damage to the sensory neurons so that neurogenic inflammatory mediators are no longer synthesized, meaning that the neurogenic response is permanently abolished.

4.2. Substance P. SP is an 11 amino acid peptide that is a member of the tachykinin family, so named for their fast-acting properties [55], which also includes neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK), and neuropeptide γ (NPγ), amongst others. SP is released from both central and peripheral endings of primary afferent neurons where it functions as a neurotransmitter [41, 55]. SP, along with other tachykinins, is produced from the preprotachykinin (PPT) A and B genes. Alternate splicing of the PPTA gene yields the α- and δ-transcripts giving rise to SP, NKA, NPK, and NKγ, whereas the β- and γ-transcripts only produce SP. The PPTB gene only encodes for NKB. SP synthesis occurs at the cell body ribosomes, where it is then packaged into vesicles and axonally transported to the terminal endings for final enzymatic processing [56]. Precursor proteins are stored in secretory granules along with processing enzymes for posttranslational modifications and release of the active peptide [39, 57]. The biologically active peptide is then stored in large, dense vesicles ready for release. Under normal conditions, substantial amounts of SP are synthesised and stored within neurons [56]. However, activation or damage of these neurons results in the rapid release of SP and other neuropeptides [39].

SP is widely distributed throughout the central and peripheral nervous systems, with α-PPTA transcripts more abundant within the brain and β-PPTA transcripts more abundant in peripheral tissues. Specifically, in the brain, SP immunoreactivity has been demonstrated in the rhinencephalon, telencephalon, basal ganglia, hippocampus, amygdala, septal areas, diencephalon, hypothalamus, mesencephalon, metencephalon, pons, myelencephalon, and spinal cord. SP has also been found localized within brain endothelial cells and microglia [58–60]. In peripheral tissues, SP and other sensory neuropeptides are distributed throughout the gut, respiratory system, urinary system, immune system, blood, and blood vessels [37]. SP is localized in capsaicin sensitive neurons and is released from central and peripheral endings of primary afferent neurons in response to various noxious stimuli [39]. Of interest is the fact that SP is colocalized with other classical transmitters such as serotonin and glutamate, and other neuropeptides such as CGRP and NKA [56, 59].

Once released, SP may be cleared and inactivated by many different proteolytic enzymes including neutral endopeptidase (NEP) [61, 62] and angiotensin-converting enzyme (ACE) [61, 63, 64], amongst others. Both NEP and ACE catalyse the degradation of the hydrolytic bonds of SP, rendering it inactive without the carboxyl terminus required to bind to its receptor [56]. Specifically, NEP has been shown to degrade SP within the brain [65], spinal cord [66], and peripheral tissues [39], whereas ACE has been shown to degrade SP in plasma, CSF, and brain, in particular the substantia nigra [67].

The biological actions of SP are mediated through its binding at tachykinin NK receptors which are rhodopsin-like membrane structures comprised of 7 transmembrane domains connected by intra- and extracellular loops and coupled to G proteins [68]. To date, 3 mammalian tachykinin receptors have been identified, the NK1, NK2, and NK3 receptors [69]. The tachykinins share a common carboxyl terminal sequence that reflects their common biological action, and, as a result, some cross-reactivity amongst tachykinin receptors

exists [70]. Each of the tachykinins may act on all receptor types with varying affinities depending upon receptor availability and neuropeptide concentration. Under normal conditions, SP has a high affinity for the NK1 receptor, NKA for the NK2 receptor, and NKB for the NK3 receptor [38, 71]. The NK1 receptor is a 403 amino acid protein that is highly conserved with only discrete variations amongst species. An NK1 autoreceptor has also been characterized to be involved in the regulation of SP release [72–75]. NK1 receptors are found in their highest levels in the caudate putamen and superior colliculus; however, they are also found in low to moderate levels in the inferior colliculus, olfactory bulb, hypothalamus, cerebral cortex, septum, striatum, mesencephalon, and dorsal horn of the spinal cord [75]. NK1 receptors are expressed by a wide variety of cell types including neurons, astrocytes, oligodendrocytes, endothelial cells, and microglia [76].

SP release is initiated in response to Ca^{2+}-dependent depolarisation of neurons, induced by a variety of stimuli including electrical stimulation, pH changes, and ligand binding to their receptors, including capsaicin [37, 57]. Once released, SP has several effects including direct postsynaptic actions as a neurotransmitter, modulatory function at postsynaptic sites, and/or paracrine functions on nonneuronal targets [57]. Transduction of the SP signal then occurs through the action of G proteins associated with the intracellular domain of the NK1 receptor, leading to an elevation in cAMP as a secondary messenger, which through a cascade of events, leads to the regulation of ion channels, enzyme activity, and changes in gene expression [48, 77]. Although normally confined to the cell membrane, the NK1-SP complex is rapidly internalised following SP binding. SP is then removed by endosomal acidification and targeted by the lysosomes for degradation, whilst the NK1 receptor is recycled to the cell membrane [57].

In addition to its role in neurogenic inflammation, SP may induce classical inflammatory reactions through the release of cytokines and recruitment of immune cells. In the skin, SP acts in a dose-dependant fashion to induce mast cell degranulation and histamine and tumour necrosis factor-α along with variable release of leukotriene B4 [78, 79]. SP also acts to induce widespread microvascular permeability. Virtually all blood vessels are surrounded by primary sensory nerve fibers that secrete SP, and the cerebral blood vessels are particularly well innervated. Intravenous injection of SP has been shown to increase the permeability of dural blood vessels as evidenced by leakage of horseradish peroxidase in association with widening of junctions between endothelial cells and an increase in the number of cytoplasmic vesicles [80].

In brain endothelial cells, the normal resting level of free Ca^{2+} is 100 nM [81]. SP causes calcium responses in the endothelial cells of the BBB of approximately 1000 nM and hence increase Ca^{2+} levels leading to increased BBB permeability through cell contraction [81, 82]. In conjunction with this, treatment with SP of cerebral capillary endothelial cells cocultured with astrocytes has been shown to decrease the concentration of ZO-1 and claudin-5 tight junctional proteins, resulting in increased permeability of the simulated BBB [83].

SP is present in cerebral capillary endothelial cells, and its secretion by these cells can be increased through treatment with high doses of cytokines, including interleukin-1β and tumour necrosis factor α [60, 84]. This increase in SP released from brain endothelial cells was found to be associated with an increase in the expression of β-preprotachykinin mRNA, a precursor for SP, inside the cells [60]. Spantide, a NK1 antagonist, reversed this increase in SP release from endothelial cells and the subsequent increased permeability of the BBB in a dose-dependent fashion [84]. Through the use of electron microscopy, it was shown that the morphological changes associated with SP interactions with endothelial cells were also neutralized [84].

SP has been implicated in the pathogenesis of many neurological diseases, due to its effects on BBB permeability. Thus, NK1 antagonists have been investigated for the treatment of chronic diseases such as Parkinson's [85], depression [86], brain tumours [43, 87, 88], and migraine [89] with variable success. However, this paper focuses primarily on acute disorders of the CNS.

The only NK1 receptor antagonist that is currently available and approved for use clinically is aprepitant. This drug is used as an antiemetic to combat chemotherapy-induced nausea in cancer patients and is generally well tolerated [90]. Thus, NK1 receptor antagonist treatment is an appealing alternative to classical anti-inflammatory drugs, the use of which are often limited by detrimental side effects for the treatment of acute and chronic CNS diseases.

4.3. Calcitonin Gene-Related Peptide. CGRP is a neuropeptide that is commonly colocalized and released with SP, particularly within sensory C fibers that innervate cerebral vasculature [91–94]. CGRP is the most potent endogenous vasodilator [95] and has been shown to increase the diameter of large cerebral arteries and arterioles. This vasodilation has been shown in many species, including the carotid arterial bed of rabbits, piglet arterioles, pial artery of cats, and guinea pigs [96–99]. Furthermore, CGRP infusion in healthy human subjects causes middle meningeal artery dilation [100]. The relaxation of blood vessels by CGRP is mediated by protein kinase C [101]. There are two isoforms of CGRP, CGRPα and CGRPβ, which are encoded by alternate RNA processing of the gene for calcitonin located on chromosome 11 and CGRPβ [102, 103]. These isoforms differ in only a single amino acid and are functionally similar, although CGRPα is the predominate form found in the CNS [104]. CGRP exerts its function through binding at the CGRP receptor, which like the NK1 receptor, has seven transmembrane domains and is coupled to a G protein. The receptor interacts with a single transmembrane receptor activity modifying protein to allow for activation to occur [105, 106]. These receptor complexes are commonly located on neurons, astrocytes, smooth muscle cells, and endothelial cells, particularly those lining dural blood vessels [107–109]. CGRP potentiates the actions of SP [110], which is thought to be through interference with SP breakdown processes [111, 112].

5. Traumatic Brain Injury

TBI results from physical trauma to the head that consequently causes injury to the brain. It is currently the leading

cause of death in individuals under the age of 45 years, with an incidence range of 100–3000 per 100,000 and death rates reported as approximately 18.4 per 100,000 [113–118]. Secondary injury, defined as the persisting alterations to chemicals, cells, and metabolism in the hours and weeks following the primary injury to the brain, is thought to be responsible for substantial cerebral edema and development of neurological deficits [119]. This is of great importance as cerebral edema has previously been shown to be a significant predictor of TBI-induced mortality [15, 18].

The majority of TBI cases can be attributed to motor vehicle accidents, motorcycle accidents, bicycle accidents, and pedestrian injuries [120]. Survivors are often are left with debilitating neurological deficits after injury [121, 122], so in addition to the enormous personal burden to victims and their families, the financial impact for the community in terms of hospitalization, treatment, rehabilitation, and specialized care runs into the billions of dollars annually. Despite improvements in motor vehicle safety measures and the marginal decrease in the mortality of trauma-related pathologies [123], TBI still has a significant epidemiological and economic burden on society [124].

Outcome following motor vehicle accident-induced trauma is superior in comparison with patients who experience CNS trauma as a result of a fall, likely due to the advanced age generally seen in people who are injured through falling and the younger demographic involved in traffic accidents [125]. Factors that have been implicated in determining the prognosis for patients include age, Glasgow Coma Scale score, arterial hypotension, computed tomography findings, and pupillary reactivity [126]. Childhood populations of trauma victims with evidence of cerebral edema on neuroimaging have shown significantly poorer outcome [15]. Despite this devastating impact, there is currently no approved therapy for the treatment of head trauma, largely because the mechanisms associated with neuronal cell death and the development of cerebral edema are poorly understood. Therefore, recent studies have focused on ameliorating cerebral edema in an attempt to improve recovery following trauma.

TBI results from acceleration/deceleration forces that produce rapid movement of the brain within the skull, or from the head impacting with an object form. The type and severity of the resultant injury are dependent upon the nature of the initiating force, in addition to the site, direction and magnitude of the impact. Injury to the brain following TBI may arise from two different mechanisms, designated as primary and secondary injuries. Primary injury is irreversible, occurring at the time of impact and encompassing the mechanical forces at the time of injury that damage blood vessels, axons, neurons, and glia through shearing, tearing, and stretching [127]. It also includes surface contusions and lacerations, diffuse axonal injury, and hemorrhage. The shearing forces applied to neurons in response to injury cause massive ion fluxes across neuronal membranes, resulting in the widespread loss of membrane potential and the excessive release of neurotransmitters [128]. Such cellular events are part of an evolving sequence of cellular, neurochemical, and metabolic alterations termed as secondary injury, which is initiated by the initial traumatic events and ensues in the hours to days following the initial traumatic event. Secondary injury has profound effects on ion channels, membranes, intracellular biochemical events, and second messenger systems and includes changes in neurotransmitter release, ion homeostasis, blood flow, and cellular bioenergetic state, along with oxidative stress and lactoacidosis [129]. Infiltration of the brain and perilesional area by neutrophils, macrophages, and microglia is also a characteristic of secondary injury and inflammation [130]. Unlike primary injury, such secondary injury is potentially reversible, because its delayed nature provides a therapeutic window for pharmacological intervention. The aim of such therapy is to reduce injury and improve both outcome and survival. However, despite the large number of experimental studies successfully targeting individual injury factors, none have resulted in an effective therapy that can be used clinically.

Substance P in Traumatic Brain Injury. Traumatic brain injury is associated with significant edema formation, proposed by our own group to be mediated by SP and thus neurogenic inflammation. In the human postmortem tissue, SP immunoreactivity is increased following traumatic brain injury [131]. Similarly, perivascular SP immunoreactivity was increased in a rat model of brain trauma, which was closely associated with increased Evans blue leakage into the neuropil, commonly used as an exogenous marker of increased BBB permeability [132]. Animals chronically pretreated with capsaicin, an agent shown to deplete neuropeptides, significantly reduced BBB permeability, cerebral edema, and functional deficits as compared to vehicle-treated controls in a rodent model of diffuse traumatic brain injury [133]. Likewise, NK1 antagonist treatment has been shown to reduce BBB permeability and cerebral edema and to improve functional outcome in this model [132, 134]. Similarly, this treatment has also resulted in amelioration of the proliferative microglial response to diffuse traumatic brain injury [135]. Prevention of SP breakdown with ACE inhibitor treatment also resulted in increased evidence of trauma-induced histological damage and exacerbation of neurological deficits [136].

Most of these studies investigating the effects of NK1 antagonist treatment following diffuse traumatic brain injury have been performed in male rats. This is because estrogen may provide additional neuroprotection in females, which could confound experimental results. However, it is important that drugs to treat the complications following traumatic brain injury be effective in both sexes. Recently, an NK1 antagonist treatment has been investigated in an experimental model of trauma in female rats and has been shown to similarly reduce BBB permeability and cerebral edema following injury [137].

Together, these findings make a strong argument for links between elevated perivascular SP and increased BBB permeability leading to cerebral edema formation following both experimental and clinical traumatic brain injuries. Therefore, NK1 antagonist treatment may be beneficial for patients with traumatic brain injury in relieving symptoms of cerebral edema and improving recovery.

6. Traumatic Spinal Cord Injury

Spinal cord injury (SCI) is an insult to the spinal tissue that results in altered motor, sensory, and autonomic functioning. The incidence and mortality estimates for SCI range from 1.3 to 8.3 per 100,000 and 0.3–1.8 per 100,000, respectively, which is approximately 10% of the rates reported for TBI [138–140]. Common mechanisms of SCI are vertebral dislocation and burst fracture injury [141]. Similar to TBI, initial primary injuries including laceration of blood vessels, bone fracture, and axonal injury, are followed by persistent secondary inflammatory processes. Specifically, this commonly includes immune cell accumulation and inflammatory mediatory release, which have been linked to BSCB disruption [142, 143]. The BSCB controls the passage of substances between capillaries and spinal tissue and is disrupted to cause vasogenic edema [144]. This increased permeability of the BSCB may be evident over several segments rostral and caudal from the injury epicenter, particularly following severe spinal cord injury [25, 26]. The importance of this process is illustrated by the established link between edema formation and SCI-induced mortality [16].

Nearly 80% of spinal trauma occurs in males, with two peak age groups affected, young adults in their 20s and the elderly over 60 years of age. This bimodal demographic is thought to be associated with traffic accidents and falls, respectively [145]. Brain injury is a common comorbidity for spinal trauma, which is unsurprising as it has many common epidemiological features. The most common site of traumatic spinal cord injury is the cervical level, with decreasing incidence in the lumbar and thoracic regions of the cord [146]. The clinical deficits increase in severity as the SCI occurs at a higher, or more superior, level.

Spinal cord injury is a highly inflammatory process, resulting in immune cell chemotaxis. In a rodent model of thoracic contusion, inflammatory cytokine release was evident in the spinal cord following injury, which replicates the human condition where a similar pattern of cytokine expression was evident in the CSF, although at a later time point [147]. Additionally, following T9 spinal contusion, neutrophil, macrophages/microglia, and T cells infiltrate the injured spinal cord and remain evident up to 180 days following trauma [148].

The promising research on the role of SP in edema development following brain trauma has led researchers to consider that the pathogenesis of secondary injury following spinal cord injury may have similar mechanisms. Moreover, it is thought that this injury type may too respond to manipulation of inflammatory neuropeptides, as it has previously been shown that resolution of BSCB permeability and edema results in improved functional outcome in animal models of SCI [149, 150].

Neurogenic Inflammation in Spinal Cord Injury. Previous studies have shown that SP expression is altered following traumatic SCI in both the human condition and in experimental animal models. In a combined human cohort of both peripheral nerve and SCI patients, increased SP levels in the cerebrospinal fluid were observed in comparison with control patients [151]. Similarly, at both 1 and 2 hours after focal thoracic injury, there was a significant increase in SP found up to 5 mm from the site of injury [152]. In addition, there was a significant increase in brain SP 5 hours after injury [152]. Therefore, the modulation of SP following trauma to the spinal cord may occur throughout the entire CNS. There was also an increase in SP evident following T12 transection of the spinal cord in female cats [153]. In contrast, a weight drop model of trauma in rodents resulted in decreased SP at the site of injury [154]. Furthermore, NK1 receptors have been shown to be significantly increased 1 week after injury using a rat model of thoracic cordotomy [155]. The alterations in both SP and NK1 receptor expression in the spinal cord following trauma suggest that SP may play a part in the pathogenesis of spinal cord injury and its complications. However, further studies are required to determine its exact role.

There has been limited research on the role of CGRP in traumatic spinal cord injury. It has been shown, following either C4 or T13 hemisection, that primary afferents axons immunostaining for CGRP grow into the area of injury [156, 157]. However, the functional or mechanistic significance of this is yet to be elucidated. Therefore, the evidence for a role of CGRP and possible therapeutic benefit following its manipulation is far less compelling for spinal injury when compared to the results seen for other pathologies.

7. Stroke

Stroke is the third most common cause of disability-adjusted life years and as such is a major health problem worldwide [158]. Specifically, a staggering 15 million people worldwide suffer a stroke each year, of which 10 million either die or are left permanently disabled [159]. The social and economic costs of stroke are consequently enormous. Despite this, there is currently only one approved treatment for use in stroke, that being tissue plasminogen activator within 4.5 h of symptom onset [160]. However, as little as 5–15% of stroke patients are eligible for and receive such treatment. In the case of hemorrhagic stroke, little can be done beyond evacuation of the hemorrhage if surgically accessible. As such, novel therapies that can limit or reverse ischemic injury following stroke are urgently required.

Stroke is defined as an interruption in the cerebral blood flow of vascular origin that restricts the supply of vital oxygen and substrates for neurons. Stroke can be broadly classified into two types, ischemic and hemorrhagic. Ischemic stroke most frequently involves a thrombus (local origin) or embolus (distant origin) obstructing blood flow, although when blood flow is reestablished, reperfusion injury may occur. This involves the interaction of blood with oxygen-deprived tissue resulting in substantial inflammation and oxidative stress. Hemorrhagic stroke refers to a bleed within the brain. In both instances, cerebral ischemia results, and if blood flow is not rapidly restored, death of cells may result with associated long-term functional deficits [161]. Restoration of blood flow is seen as an urgent priority in reducing the extent of tissue injury and preserving function. However, it is now well accepted that secondary injury processes continue to evolve many hours to days following stroke and also

contribute to the size of the infarct [162]. With respect to outcome, hemorrhagic stroke generally has a poorer outcome than ischemic stroke with mortality rates in the order of 37% and 11%, respectively [163]. Hemorrhagic stroke may be classified as either intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH). The rupture of charcot-bouchard microaneurysms on small arterioles commonly leads to ICH, whereas ruptured berry aneurisms within the Circle of Willis are often the cause of SAH [164, 165].

Following stroke, the resultant tissue injury and infarction can be considered as being made up of two components, the infarct core and the surrounding penumbral tissue [166]. The infarct core is widely considered to be irreversibly damaged during ischemic stroke, with cell death occurring rapidly within this region. In the penumbral tissue, however, blood flow is less restricted and so there exists an opportunity for neuronal tissue to survive the insult. Nevertheless, cell death may continue to occur here as a result of secondary biochemical and physiological mechanisms that manifest over the hours to days following stroke [162, 166]. Similar to TBI, there are diverse arrays of secondary injury processes that contribute to injury and cell loss following stroke, including excitotoxicity, oxidative stress, inflammation, apoptosis, increased vascular permeability, and cerebral edema, amongst others [167]. Given the delayed nature of secondary injury following stroke, there is an opportunity for pharmacological intervention to limit tissue damage and cell death.

Both SAH and ICH can often result in rapid death, meaning that there is only a small window for therapeutic administration or surgical intervention. Furthermore, given that the mass effect of such hemorrhagic lesions is substantial, the contribution of secondary injury processes to functional impairments is smaller compared with ischemic lesions. In contrast, ischemic stroke has a pattern of injury more comparable to TBI, with increased permeability of the BBB and cerebral edema as common features. Mortality rates increase with time following stroke, demonstrating that even if patients survive the initial insult, the condition may still be fatal due to persistent secondary injury mechanisms such as the development of cerebral edema [168]. The type and severity of edema may be influenced by the duration and severity of ischemia and reperfusion status, amongst other factors, and may also differ between the core and the penumbra of the stroke lesion.

Cerebral edema is a major cause of clinical deterioration within the first 24 h, is the leading cause of death within the first week, and is a predictor of poor outcome following stroke [30]. Clinical studies report that it is maximal between 1 and 3 d following stroke [18], whilst experimental studies report its presence as early as 15 mins after the onset of vascular occlusion [169]. The presence of vasogenic edema is of particular concern, not only because it increases brain volume, but also because in the setting of vascular recanalization, it increases risk of hemorrhagic transformation from damaged blood vessels and excess fluid accumulation [170].

7.1. Substance P in Stroke. To date, few groups have investigated SP in cerebral ischemia [171], and only our research group has explored the role of neurogenic inflammation following stroke [172–175]. Our own studies have recently shown that SP is increased following experimental ischemic stroke, indicative of neurogenic inflammation. Specifically, increased SP immunoreactivity was observed within penumbral tissue at 24 h following stroke, being particularly marked in perivascular tissue. Such an increase in SP was confirmed through SP ELISA of the ischemic hemisphere [174]. The increase in SP was associated with marked disruption to the BBB, as evidenced by increased Evan's blue extravasation into the brain parenchyma at 24 h after stroke, thus, supporting previous observations of a delayed opening of the BBB [176]. The increased BBB permeability was observed in the setting of profound cerebral edema, suggesting that the edema had a vasogenic component [174]. Furthermore, profound and persistent functional deficits with respect to motor, sensory, and neurological function were observed [174].

A role for SP in clinical stroke has also been documented by Bruno and colleagues [177], suggesting that there may be a role for neurogenic inflammation in this disease pathogenesis. They observed that patients with transient ischemic attack and complete stroke showed elevated serum SP when compared to the control group [177]. Interestingly, individuals with transient ischemic attack showed a greater elevation than complete stroke [177].

Early studies reported that hypoxia of the rabbit carotid body increased SP release as a function of the severity of the hypoxic insult [178]. This finding suggested that SP release may be a tissue response to hypoxia/ischemia. Consistent with this, capsaicin pre- or posttreatment was shown to confer protection from neonatal hypoxia-ischemia injury with a reduction in infarct volume and apoptosis, in addition to improved vascular dynamics [179].

Given the clear increase in SP that has been documented in both experimental and clinical stroke studies, NK1 tachykinin receptor antagonists have been investigated for their potential utility in reducing BBB dysfunction and vasogenic edema in the setting of ischemic stroke. Yu and colleagues [171] reported a reduction in infarct volume and an improvement in neurological outcome as measured at 24 h poststroke following administration of the NK1 tachykinin receptor antagonist SR-14033. Recently, our group has extended these initial observations and extensively characterized the effect of NK1 tachykinin receptor antagonist treatment in experimental ischemic stroke. Specifically, we have shown that intravenous NK1 antagonist treatment administered 4 hours following stroke resulted in decreased evidence of cerebral edema [174]. Furthermore, when combined with the current standard clot dissolution treatment, tissue plasminogen activator (tPA), NK1 antagonist treatment resulted in equal or better performance in functional outcome tests when compared to NK1 antagonist or tPA alone [175].

7.2. Substance P in Subarachnoid Hemorrhage. Similar to ischemic stroke, altered SP expression has been reported following SAH. Perivascular SP expression was increased in two models of SAH, injection of autologous blood into the prechiasmatic cistern and following puncture of the middle cerebral artery to cause an endogenous bleed [180]. However, NK1

tachykinin receptor antagonist treatment was unable to ameliorate the raised ICP, cerebral edema, or impaired functional outcome that resulted in either of these models of SAH [180]. A possible reason for this is that the pathogenesis of SAH differs greatly from ischemic stroke, in which NK1 tachykinin receptor antagonists have shown promise. SAH presents less opportunity for therapeutic intervention, due to the mass effect of the bleed, such that therapeutic interventions that act to modulate the permeability of BBB have limited effects. Thus, the functional deficits that result from SAH may be more related to the space occupying blood and damage from its breakdown products rather than to cerebral edema.

7.3. Calcitonin Gene-Related Peptide in Ischemic Stroke. The well-established vasodilatory actions of CGRP have led researchers to postulate that it may play a protective role to promote cerebral blood flow following ischemic stroke. This effect was demonstrated in a rat model of middle cerebral artery reperfusion stroke. Following injury, treatment with CGRP administered at the beginning of reperfusion resulted in a reduction of arterial blood pressure, decreased the infarct volume, and ameliorated the increased BBB permeability subsequently inhibiting cerebral edema formation [181, 182].

Along with the vasodilatory actions of CGRP, the mechanism of neuroprotection following ischemic reperfusion stroke may be through modulation of water channels and other elements of the BBB. As such, in two studies using the middle cerebral artery reperfusion model of rodent stroke, CGRP treatment resulted in decreased aquaporin 4 mRNA and protein expression [181, 182]. In conjunction, the reduction in tight junction proteins normally associated with stroke was ameliorated, along with reduced evidence of ultrastructural damage of endothelial cells [181, 182]. Similarly, increased expression of basic fibroblast growth factor has been found following experimental ischemic reperfusion stroke treated with CGRP, which likely acts to improve the structural integrity of the BBB basement membrane [182]. Furthermore, the neuroprotective effects of leptin in a mouse model of middle cerebral artery occlusion and reperfusion injury have been shown to be mediated by CGRP, resulting in increased blood flow and once again reduced infarct volume [183]. Thus, CGRP may be a promising treatment to improve functional outcome following cerebral ischemia through multiple actions on the BBB to reduce the severity of injury.

7.4. Calcitonin Gene-Related Peptide in Subarachnoid Hemorrhage. Akin to ischemic stroke, CGRP is thought to be beneficial following SAH. CGRP has been measured in the cranial venous outflow of 34 patients following SAH and was found to be elevated when compared to the control group, although there was no change in SP levels [184]. In contrast, following subarachnoid hemorrhage, autopsy brain concentrations of CGRP were reduced in comparison with controls in the location of the proximal middle cerebral artery [185]. Therefore, SAH results in modulation of CGRP levels in both the blood and the brain. A possible reason for the differential effects may be that the study in which CGRP was elevated was conducted on patients who had survived their SAH, whilst decreased CGRP was evident following the fatal condition. Thus, the severity of SAH may determine the extent and direction of changes in CGRP. It is postulated that exhaustion of CGRP may be involved in vasospasm, which is most common following severe SAH, and is often a fatal complication.

CGRP has been tested in both clinical SAH patients and in experimental models of SAH showing protection from abnormal blood vessel contraction. Intravenous administration of human αCGRP significantly inhibited vasoconstriction in comparison to that evident prior to infusion in 5 patients [186]. Similarly, when rabbit basilar artery strips were isolated following experimental subarachnoid hemorrhage, responsiveness to in vitro application of CGRP to induce blood vessel relaxation was impaired when compared to those from the control group [101]. This result suggests that increased CGRP levels are required in stroke patients. It is likely that CGRP treatment may hold promise for the prevention of complications associated with subarachnoid hemorrhage.

Taken together, both animal and clinical studies show that neurogenic inflammation plays an integral role in the pathogenesis of both ischemic stroke and SAH. However, there is a differential effect of inflammatory neuropeptides in these conditions. The role of neurogenic inflammation in ICH has not been widely investigated, although it is likely that, similar to SAH, the edema component of this condition may not contribute as significantly as blood volume to the development of neurological deficits. There is evidence of SP mediation of many deleterious secondary injury mechanisms following ischemic reperfusion injury, including cerebral edema formation. Thus the NK1 receptor is a promising target for pharmacological manipulation to improve patient outcomes. In contrast, SP does not seem to play a significant role in the immediate injury following subarachnoid SAH. CGRP-induced vasodilation may improve blood flow to hypoxic brain tissue during cerebral ischemia and prevent vasospasm following subarachnoid hemorrhage. This indicates that specific neurogenic inflammatory mediators need to be targeted in different ways to optimize treatment following stroke.

8. Bacterial Meningitis

Meningitis is characterized by infection and subsequent acute inflammation of the meninges that cover the outside of the brain. The most common causative infectious agent is bacteria, specifically *Neisseria meningitidis* and *Streptococcus pneumoniae*. There is a marked adult incidence of bacterial meningitis but generally children are most susceptible [187]. Meningitis is associated with CNS symptoms such as neck stiffness, headache, photophobia, phonophobia, altered consciousness, and neurological state, as well as systemic signs of inflammation such as fever, nausea, and vomiting. Additionally, individual bacteria types may be associated with specific features, for example, *Neisseria meningitidis* produces a characteristic rash.

The introduction of vaccinations against specific strains of bacteria has substantially reduced the incidence of this meningitis [188]. Despite this, the availability of antibiotics to

combat bacterial infection of the meningitis remains a medical emergency due to the close proximity of inflammation to neurological tissue. This poses a critical threat to brain tissue, not only due to the presence of bacteria, but also the contribution of secondary injury processes. Specifically, inflammatory processes are associated with increased permeability of the BBB and cerebral edema, which worsen the prognosis associated with this disease [6]. Furthermore, cytokine production and leukocyte accumulation are key features in the pathogenesis of bacterial meningitis.

Currently, anti-inflammatory agents are used in an attempt to combat the symptoms of meningitis, although the dose and duration of treatment are limited by deleterious side effects of the commonly used drugs like the synthetic corticosteroid, dexamethasone. Therefore, alternative therapeutic agents that combat secondary injury and inflammatory processes are attractive targets for investigation. Neurogenic inflammation may be a worthy target given its documented role in BBB permeability and cerebral edema in the setting of acute brain injury and stroke. Specifically, NK1 tachykinin receptor antagonists are able to block neurogenic inflammation by modulating neuropeptide action. In the setting of meningitis, this may prevent deleterious changes in diameter and permeability of cerebral blood vessels and thus leukocyte infiltration and edema formation.

8.1. Substance P in Meningitis. In vitro, SP has been shown to increase the production of inflammatory cytokines by astrocytes and microglia when exposed to *Neisseria meningitidis* and *Borrelia burgdorferi* gram-negative bacteria [189]. Similarly, SP treatment of microglia in vitro, which were exposed to the gram-negative *Borrelia burgdorferi* bacteria, results in augmented secretion of prostaglandin E2 [190]. This effect was ameliorated by NK1 tachykinin receptor antagonist treatment and in NK1 knockout cell lines [190]. Furthermore, microglial cells respond to the presence of the gram-positive bacteria *Streptococcus pneumoniae* with upregulation of NK1 receptors by this cell type [191]. These results suggest that NK1 antagonist treatment may act to inhibit many inflammatory processes associated with bacterial meningitis that cause substantial tissue damage and worsen outcome.

Positive results from in vitro studies led to in vivo experiments to determine the effectiveness of NK1 receptor antagonists in experimental mouse models of both gram-positive and gram-negative bacterial meningitis. Intracerebral inoculation of *Neisseria meningitidis* and *Borrelia burgdorferi* into C57BL/6 mice resulted in increased inflammatory cytokine and decreased immunosuppressive cytokine secretion, resulting in a substantially proinflammatory environment [189]. Correspondingly, intracerebral inoculation of female C57BL/6 mice with *Streptococcus pneumoniae* caused a similar pattern of cytokine expression along with gliosis, demyelination, and increased BBB permeability [191]. These features were abolished with both NK1 antagonist treatment and in NK1 knockout mice [189, 191]. Therefore, NK1 antagonist treatment may be able to limit infection-associated inflammation and subsequent edema formation through its ability to inhibit inflammatory cytokine secretion and modulate the permeability of the BBB. The results suggest that in the future, this class of agents could be used as an alternative to classical anti-inflammatory drugs like dexamethasone. However, the effect of NK1 receptor antagonist treatment has only been demonstrated in experimental animal models of meningitis; thus, further investigation into the role of SP in the human condition is required.

8.2. Calcitonin Gene-Related Peptide in Meningitis. The proinflammatory nature of meningitis makes CGRP a likely candidate in the pathogenesis of associated vascular changes, although there has been limited investigation into this area. Nevertheless, patients with acute bacterial meningitis and sepsis have shown evidence of increased CGRP in arterial blood samples [192]. Therefore, the possible role of CGRP in the inflammatory response of bacterial meningitis warrants additional examination.

9. Conclusion

Acute injury to the brain and spinal cord is associated with a number of deleterious secondary injury processes of which altered vascular permeability and tissue swelling are of particular concern. This is further compounded by the lack of effective therapies. However, the inhibition of neurogenic inflammation may provide a novel alternative therapy for the treatment of barrier dysfunction and tissue swelling in the setting of acute CNS injury. Experimental studies of TBI and stroke have shown that blocking the action of SP with an NK1 tachykinin receptor antagonist produces profound reductions in BBB permeability, cerebral edema, and functional deficits. Studies of NK1 tachykinin receptor antagonists in SCI, meningitis, and hemorrhagic stroke are ongoing, but early results suggest that neurogenic inflammation does play a role in these pathologies, albeit a less pronounced role than in TBI and stroke. CGRP may be another worthy target alongside SP with experimental models of both hemorrhagic and ischemic stroke models showing benefits of CGRP treatment. Further investigations on the role of neurogenic inflammation and the neuropeptides SP and CGRP in the barrier dysfunction and tissue swelling that are associated with acute brain and spinal cord injury are ongoing, and given the encouraging results to date, they are certainly warranted.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

The authors would like to thank Tavik Morgenstern for the creation of artwork for this paper.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 715645, 9 pages
http://dx.doi.org/10.1155/2013/715645

Review Article

Curbing Inflammation in Burn Patients

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Received 24 February 2013; Revised 24 April 2013; Accepted 26 April 2013

Academic Editor: David A. Hart

Patients who suffer from severe burns develop metabolic imbalances and systemic inflammatory response syndrome (SIRS) which can result in multiple organ failure and death. Research aimed at reducing the inflammatory process has yielded new insight into burn injury therapies. In this review, we discuss strategies used to curb inflammation in burn injuries and note that further studies with high quality evidence are necessary.

1. Introduction

Trauma resulting from severe burn injury triggers a systemic inflammatory response syndrome (SIRS) and serious metabolic disturbances. One of the best known systemic manifestations observed in the first hours after a major burn injuries is related to increased systemic capillary permeability with protein leakage into the interstitial space, generalized edema, and a tendency toward hypovolemic shock. Adequate fluid replacement is mandatory in the first hours after a traumatic burn. However, in burn patients, other systemic disorders are also accompanied by SIRS such as cardiac dysfunction, acute respiratory distress syndrome, acute renal failure, increased intestinal permeability resulting in bacterial translocation, hypermetabolism, hypercatabolism, and sepsis [1–4]. These intense disruptions in body's homeostatic balance may result in multiple organ failure and death. Therefore, research seeking new mechanisms by which to attenuate inflammation after severe burn injury is needed. In this review, we address and discuss the available options.

2. Burns and Inflammation

Burn injury induces global changes to the entire immune system resulting in suppressed immune function and increased susceptibility to infection. This immunopathological response can contribute to the development of SIRS and subsequent multiple organ failure. Patients with severe burns are more likely to die from sepsis due to the massive release of inflammatory mediators from the burn wounds. Total body surface area (BSA) involved and smoke inhalation are predictors of death. Each one percent increase in total body surface area burned was associated with a six percent increase in mortality risk. Also, the presence of smoke inhalation increased mortality risk by ninefold [5]. In addition, the depth of the burn also affects mortality risk as full thickness burns have a poorer prognosis compared to partial thickness.

Nevertheless, the systemic disorders observed in the first hours after a severe burn injury are related to increased systemic capillary permeability with protein leakage and a tendency toward hypovolemic shock. Burns greater than 10% BSA in children or 15% BSA in adults are potentially life-threatening injuries (because of the risk of hypovolemic shock) and should be treated with formal fluid resuscitation and monitoring in a burn unit [6]. Hence, adequate fluid replacement is mandatory in the first 24 hours after the severe burn trauma minimizing the possibility of hypovolemia and early renal insufficiency. The patient with extensive burns will undergo surgery only after appropriate fluid resuscitation, which usually occurs after 48–72 hours. However, fluid resuscitation must be undertaken judiciously as excess fluids may worsen the prognosis of burn patients and care must always be present to restrict the supply of liquid to only what is necessary.

3. Volemic Resuscitation and Inflammation

3.1. Fluid-Restrictive Strategies. Despite the convenience of using formulas as an initial guide for fluid replacement (i.e., the Parkland formula: 4 mL ∗ weight (kg) ∗ % BSA), it is difficult to carry out fine adjustments in fluid delivery to the severely burned patient in practice. Commonly, there is a tendency to administer an oversupply of fluid (fluid creep) [7–9]. But what degree of excess crystalloid hydration leads to systemic complications after burns? Increasing evidence has demonstrated that aggressive crystalloid-based resuscitation strategies are associated with cardiac and pulmonary complications, gastrointestinal dysmotility, coagulation disturbances, and immunological and inflammatory mediator dysfunction. Numerous investigators have evaluated potential risk factors for developing abdominal compartment syndrome and have universally noted the excessive use of crystalloids as the primary determinant [10–12]. In our experience, we have observed that elevated levels of creatinine associated with disturbances in renal function occur concurrently with the initial signs of abdominal compartment syndrome, even without evidence of sepsis. After careful fluid restriction and diuresis induction, generalized reduction of edema is observed along with normalization of renal function. Also, disturbances in cell volume disrupt numerous regulatory mechanisms responsible for keeping the inflammatory cascade under control [10, 11].

In the last decade, our burn center staff has preferred the use of the formula: 3 mL ∗ weight (kg) ∗ % BSA of crystalloid infusion instead of the Parkland formula in the first 24 hours after burn injury. Using our formula, we have observed only minor amounts of general edema in the first days after extensive burn trauma, with consequent reduced morbidity and faster recovery. Our findings, "data not shown," have been shared by other authors [10, 13]. Fluid-restrictive strategies have been associated with a decreased frequency of and shorter time to recovery from acute respiratory distress syndrome and trends toward shorter lengths of stay and lower mortality [10, 13]. The proper control of liquids provides the ability to perform surgery earlier in patients with severe burns, thus accelerating healing.

4. Inflammation Related to the Wound

The lipid protein complex (LPC) released from burnt skin is responsible for the profound immune suppression associated with major cutaneous burns [14, 15]. Thermal injury represents a pathophysiological condition in which hyperactive macrophages are primed to stimulate the downregulation or upregulation of certain inflammatory cytokines [16–18]. Abnormal levels of proinflammatory mediators, such as tumor necrosis factor alpha (TNF-α), interleukin-1b (IL-1b), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-10 (IL-10), have been reported both systemically and locally in burn patients. It has been proposed that there is a genetic influence on cytokine production and susceptibility to sepsis. A recent and interesting study indicates that genetically determined individual differences in IL-10 production might influence the susceptibility to septic complications in burned patients [19].

Furthermore, attempts to understand immune responses in different burn depths may produce knowledge about the pathophysiology of major burns [20]. Sakallioglu et al. showed that the circulating levels of the proinflammatory cytokines, IL-6 and interferon-gamma (IFN-γ), were higher in rats with full thickness burns as compared to rats with only partial thickness burns, one hour after burn injury. The authors suggested that early elevation of IL-6 and IFN-γ can prolong inflammation in full-thickness burns [20].

Thus, the rationale for early excision of burns is the decrease in release of inflammatory mediators and bacterial colonization of wounds. This, in turn, can attenuate SIRS and reduce the occurrence of metabolic derangements, sepsis, and multiorgan failure [21]. When performed early, excision and immediate wound closure has been shown to improve survival and decrease length of hospital stay in burn patients [21].

4.1. Escharectomy. The concept of early excision of burned tissue was developed by Janzekovic in 1970, when she enumerated several advantages of this concept including removal of tissue before bacterial colonization (from 3 to 5 days after burn trauma); patients in better physical condition; improved scar quality; fewer contractures; shorter hospital stay; fewer dressing changes [22]. Skin burns result in an intense inflammatory response; thus, it has been proposed that early surgical removal of burned tissue might limit the increased production of inflammatory mediators.

In rats, escharectomy while the animal is still in shock can inhibit the overexpression of both early and late inflammatory mediators and maintain the balance of pro-/anti-inflammatory responses, thereby improving multiple organ function following severe burns [23]. Additionally, in humans, escharectomy has an immunomodulatory effect on the inflammatory mediators and reduces insulin resistance induced by major burns [24]. Moreover, both thermal injury and escharectomy induce endothelial progenitor cells (EPCs) production. EPCs migrate to sites of neovascularization in response to mediators contributing to wound healing [25]. Nevertheless, Han et al. showed a limited immunomodulatory effect of escharectomy on the inflammatory mediators in systemic inflammatory responses in burns which varied based on the extent and timing of surgery [26]. They reasoned that, in their study, only the samples taken from survivors were used for the final analysis and perhaps those patients experienced lower levels of postinjury stress.

In 1992, our burn center, Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo-HC-FMRP-USP-Brazil, changed the standard of care in severe burns from delaying surgery until after eschar separation is observed to early excision and grafting; as a result, we observed a reduction in the mortality rate from 12% to less than 5% (Table 1).

Although early surgery is well established as essential in reducing inflammation and promoting better wound healing,

Table 1: Mortality rate in the burn unit of HC-FMRP-USP-Brazil from 1992 to 2012.

Year	Survivors	Nonsurvivors	Total no. of cases	Mortality rate (%)
1992	98	14	112	12.5
1993	107	13	120	10.8
1994	83	8	91	8.8
1995	97	6	103	5.8
1996	142	5	147	3.4
1997	123	9	132	6.8
1998	141	9	150	6.0
1999	151	6	157	3.8
2000	144	6	150	4.0
2001	139	4	143	2.8
2002	142	5	147	3.4
2003	142	1	143	0.7
2004	118	11	129	8.5
2005	161	5	166	3.0
2006	178	8	186	4.3
2007	197	8	205	3.9
2008	246	10	256	3.9
2009	245	5	250	2.0
2010	217	10	227	4.4
2011	193	7	200	3.5
2012	208	3	211	1.4

other studies have reported the use of drugs to assist the modulation of inflammation after extensive and deep burns.

4.2. Antibodies Targeting Growth Factors. Recently, Sun et al. [27] investigated the effects of topically inhibiting two of these proinflammatory mediators: TNF-α and IL-6. They concluded that topical application of antibodies targeting TNF-α was effective in preventing progression of necrosis in a partial-thickness rat burn model. However, there is still clearly an increased risk for infection at injury sites and the dose-response behavior will be critical for evaluating the potential clinical efficacy of these materials.

4.3. Glucan Phosphate. Glucan phosphate (glucan), a soluble polysaccharide immunomodulator, is a modified water-soluble [beta]-1,3-linked glucose polymer derived from the cell wall of the yeast, *Saccharomyces cerevisiae* [28, 29]. Glucan phosphate treatment attenuates burn-induced inflammation and increases resistance to *Pseudomonas aeruginosa* burn wound infection in an experimental model of burn injury. Lyuksutova et al. [30] observed that intraperitoneal injection of glucan phosphate (40 mg/kg) improved survival in mice exposed to *Pseudomonas aeruginosa* in mice with 35% total BSA thermal injuries. Improved survival correlated with lower bacterial burden in the burn wound, attenuated production of proinflammatory cytokines, and enhanced production of Th1 (T helper 1) cytokines. These studies show that glucan phosphate treatment attenuates burn-induced inflammation and increases resistance to *Pseudomonas aeruginosa* burn wound infection in an experimental model of burn injury [30].

4.4. Ulinastatin. Ulinastatin is a protease inhibitor obtained from human urine; it has been shown to have anti-inflammatory effect by suppressing the production of proinflammatory cytokines [31]. Luo et al. found that treatment with ulinastatin (40,000 U/kg) could attenuate the systemic inflammatory response and visceral vasopermeability both *in vivo* and *in vitro* and may serve as a therapeutic agent for the prevention of a systemic inflammatory response and leakage of fluid into tissue after major burn injuries [32].

5. Inflammation and Coagulopathy

Coagulation and inflammation may interact during thermal injuries with major consequences for the pathogenesis of microvascular injury and subsequent multiple organ dysfunction or failure [33]; their interaction may also place patients at higher risk for development of septic complications [34]. The activation of inflammation and coagulation cascade in septic burn patients can ultimately lead to increased mortality [35]. The coagulopathy seen in burn patients is associated with the marked depletion of the endogenous regulators of the coagulation system [36–38]. Coagulation system dysfunction during the early postburn period is characterized by activation of procoagulation pathways, enhanced fibrinolytic activity, and impairment of natural anticoagulant activity. Both the thrombotic and fibrinolytic pathways are triggered proportionally to the extent of the burn [35, 36, 39, 40].

Treatment focused on the normalization of coagulation and the inhibition of systemic inflammation might have a positive impact on organ function and overall outcome in septic burn patients [34]. Molecular pathways contributing to inflammation-induced activation of coagulation and modulation of inflammation by coagulation factors have been reported in the literature over the last decade [35–38]. The activation of the thrombomodulin-protein C pathway plays a central role in the pathogenesis of acute traumatic coagulopathy [41]. Activated protein C (APC) is an important physiological anticoagulant derived from protein C by the action of the thrombomodulin-thrombin complex on endothelial cells [42]. Factor VIIa serves an important role both in the initiation of coagulation and in the activation of platelets [43]. Treatment strategies using antithrombin, protein C, and recombinant factor VIIa are based on early and continuous assessment of the bleeding and coagulation status of burn patients. The routine use of human recombinant activated protein C in burn patients with severe sepsis or septic shock is not recommended because there is a need for a large scale clinical trial to assess the benefits and harms of activated protein C in burn patients [34]. Administration of recombinant factor VIIa in acutely bleeding burn patients is recommended only if other (conventional) therapeutic options are not effective since the safety and the effectiveness

of recombinant factor VIIa in burn patients have not been established in a randomized control trial [34].

Treatment of coagulation abnormalities is a challenge for burn specialists. Clear indications, efficacy, and the economic feasibility of the use of specific coagulation factors in burn patients should be targeted in clinical trials over the next few years.

6. Inflammation, Nutrition, and Hormones

Nutritional support plays an undisputed part in the treatment of critically ill patients. Enteral nutrition supplemented with arginine, for example, alters intestinal homogenates from a pro- to an anti-inflammatory profile in mice with large areas of burn injury [44]. Nutritional support must be individually tailored in terms of quantity and quality; thus, adapting the support to requirements appears as a priority of nutritional assessment [45].

Studies have shown that increased systemic retinol binding protein (RBP) levels are associated with insulin resistance (IR) and hyperinflammation in diabetic and obese patients. Increased RBP levels occurring after burn injury correlate with increased IR, inflammatory and catabolic responses, incidence of multiorgan failure, and mortality [46]. Insulin decreases mortality and prevents multiorgan failure in critically ill patients. Jeschke et al. reported that insulin administration decreased proinflammatory cytokines and proteins while increasing activation of an anti-inflammatory response in children with burn injuries [47].

SIRS is associated with a debilitating systemic hypercatabolic state, which is mediated by cytokines and chemokines [48]. Systemic inflammatory and hepatic acute-phase responses contribute to hypermetabolism, multiorgan failure, and mortality.

One protein that has been studied in inflammation control is leptin. Leptin is a circulating hormone that regulates energy intake and expenditure, including appetite and metabolism. It is well established that leptin is involved in the regulation of inflammation [49–51]. Leptin can exert a direct effect on T cells and monocytes, causing the release of cytokines. It may also induce angiogenesis or influence angiogenic factors. Cytokines and leptin are increased in severely burned patients, including those cases associated with sepsis and those patients who ultimately do not survive their injuries while basic fibroblast growth factor (bFGF) and transforming growth factor alpha (TGFα) levels are lower in severe cases. These variations in cytokine levels may indicate impaired healing in severe burn injury patients which leads to their poorer prognosis.

Leptin production is acutely increased during infection and inflammation, as a part of the host's acute phase response [52]. On the other hand, in a thermal injury model in rats, exogenous leptin reduced microscopic damage scores in the liver, stomach, colon, and kidney and also reduced death of mononuclear cells and granulocytes. It has been suggested that leptin may diminish burn-induced inflammation and associated multiple organ failure [53]. It is possible that endogenous elevation of leptin levels during burn injury is not sufficient to enhance healing and avoid organ damage. Leptin can be regarded as a novel treatment modality to diminish burn-induced inflammation, reduce postburn immune dysfunction, and enhance burn healing [54].

Another hormone that should be mentioned in inflammation in burns is cortisol. Although hypercortisolemia has been suggested as a primary hormonal mediator of whole-body catabolism following severe burn injury, it may not play a central role in the postburn hypermetabolic catabolic response [55].

7. Oxidative Stress and Inflammation

Oxidative stress has been documented in burn injuries in both animals [56] and humans [57]. Increased free radical levels can potentiate the clotting process, aid wound reepithelization, promote angiogenesis, and influence the bactericidal ability of neutrophils and macrophages [58]. However, the oxidants must be detoxified to prevent damage to the host cells [59], a process that requires a delicate balance between oxidants and antioxidants in biological systems [60].

A patient's vitamin status is directly involved in inflammation, antioxidant response, burn wound healing, and the expected immune responses [61]. Adjuvant administration of high-dose vitamin C during the first 24 h after thermal injury significantly reduced resuscitation fluid volume requirements, body weight gain, and wound edema in humans [62, 63]. Our group recently evaluated vitamin status as it related to inflammatory and oxidative stress markers in adult patients up to three days after thermal injury. This prospective study detected decreased serum levels of vitamin C in burn patients [64]. The low levels of vitamin C can be explained by augmented cutaneous loss of ascorbic acid. Moreover, large vitamin C expenditure may have taken place in extracellular compartments, neutralizing free radicals and aiding regeneration of vitamin E, which protects the cell from lipid peroxidation [65]. In this context, there is evidence of the clinical benefits of parenteral vitamin C administration in oxidative stress conditions [66].

7.1. Methylene Blue (MB): A Selective Inhibitor of Guanylate Cyclase and Nitric Oxide Synthase (NOS) Inhibitors. It is known that severe burns can be accompanied by vasoplegic syndrome (VS), a phenomenon which is characterized by persistent and diffuse vasodilation, hypotension, and low vascular resistance resulting in circulatory and respiratory failure. The decrease in systemic vascular resistance observed in VS is associated with excessive production of nitric oxide (NO). The plasma NO content is increased during the first hours after burn injury. It seems that the increased concentration of NO, combined with other biochemical phenomena of the systemic inflammatory response, leads to a widespread leakage of protein and intravascular fluid into the interstitial space, resulting in various degrees of edema and hypovolemia. Nitric oxide stimulates soluble guanylate cyclase to increase cyclic guanosine monophosphate (cGMP) production, leading to smooth muscle relaxation. The NO competitor, methylene blue (MB), which is an inhibitor of

the soluble guanylate cyclase (sGC), has been proposed in the treatment of refractory cases of vasoplegia. We suggest MB as a viable, safe, and useful coadjuvant therapeutic tool to be used during fluid resuscitation [67, 68].

Another way to suppress vascular hyperpermeability is the administration of nitric oxide synthase (NOS) inhibitors [69]. However, NOS inhibitors are not currently in clinical use due to their lack of specificity, as they carry the consequent risk of generalized tissue necrosis. For these reasons, it seems more appropriate to use MB as a therapeutic agent in the aforementioned shock-related vasoplegic states. Methylene blue does not interfere with NOS and has played a longstanding beneficial role in many other clinical conditions. As a potent guanylate cyclase inhibitor, MP blocks the increase in cGMP levels and, consequently, prevents vascular smooth muscle NO endothelium-dependent relaxation [70]. We feel that a controlled randomized trial to assess MB as a viable coadjuvant therapeutic tool of fluid resuscitation in burn injury is a necessary next step.

8. Inflammation and Inhalation

The intrinsic mechanisms by which inhalation injury contributes to elevated mortality are unclear. One hypothesis is that a massive influx of inflammatory mediators and cells (primarily neutrophils which can release mitochondrial DNA to the extracellular space on activation) results in direct injury to the airway, leading to production of secretions and airway obstruction with mucus and products of inflammatory cell breakdown including extracellular deoxyribonucleic acid [71, 72].

Joyner et al. [72] found markedly elevated DNA levels in airway secretions in children early after burn or inhalation injury. Elevated extracellular DNA levels are correlated with the presence of cytokine markers of inflammation and injury such as IL-6, IL-8, and TGF-β1. Potential sources of extracellular DNA in airway secretions include directly damaged epithelial cells and inflammatory cells responding to injury or infection [73]. Therefore, treatment with deoxyribonuclease (DNAse) has shown some clinical benefit in terms of improvement in airways obstruction [74, 75]. These studies raise the possibility of use DNAse for treatment after burn inhalation injury.

9. Other Possibilities

9.1. Mesenchymal Stem Cell (MSC). In addition to the use of MSCs in regenerative research, it is known that these cells can also be used for modulation of inflammation. Bone-marrow-derived MSC allografts administered via intravenous transplantation have been shown to decrease proinflammatory cytokines, increase anti-inflammatory cytokines, decrease lung water mass fraction, ameliorate the systemic inflammatory response, and protect lung tissue in rabbits with smoke inhalation injury [76]. MSCs may also enhance burn wound healing and attenuate the immunosuppressant effects of the exacerbated inflammatory response and hypermetabolism in large burn injuries. Stem cell therapy may offer an additional means of mitigating burn hypercatabolism by preventing apoptosis of burned tissue. Investigation of the mechanism for this apoptotic arrest may reveal approaches toward slowing muscle breakdown associated with hypermetabolic response to burn [77, 78].

MSCs have a therapeutic benefit in burn-injured animals by providing anti-inflammatory as well as antiapoptotic effects. Human MSCs administered to the muscle of burned rats reduced infiltration of inflammatory cells into organs as kidney, lung, and liver. Interleukin-10 (IL-10) is one of the key cytokines with anti-inflammatory capacities; it has been demonstrated that MSCs can enhance the secretion of IL-10 by macrophages or dendritic cells. Furthermore, the activation of the AKT (V-murine thymoma viral oncogene homolog 1) signaling pathway in transplanted MSCs can provide an antiapoptotic effect in clinical settings of systemic inflammation [79].

9.2. Hyperbaric Oxygenation. In an experimental study in rats, treatment with hyperbaric oxygen accelerated the recovery from burn wounds and reduced the development of scars [80]. Rates of inflammation and fibrosis were lower hyperbaric-oxygen-treated animals as compared to controls. Hyperbaric oxygen maintains tissue viability by preventing microvascular tissue damage, reducing edema, and providing adequate oxygen to the damaged tissues. Hyperbaric treatment supports wound healing by reducing edema, improving microcirculation, decreasing the inflammatory response, and accelerating epithelialization. It may also reduce the progressive systemic effects of burns. Nevertheless, there is no consensus in the literature regarding the optimal timing and dosing of the hyperbaric oxygen treatment [81–83].

9.3. Hypothermia. Hypothermia has been implicated as an aggravating factor in critically ill patients, including those with serious burns [84, 85]. On the other hand, therapeutic hypothermia has been proposed to be beneficial in an array of human pathologies including cardiac arrest, stroke, traumatic brain and spinal cord injury, and hemorrhagic shock. Burn depth progression is multifactorial but inflammation plays a significant role. Systemic hypothermia decreased burn depth progression in a rodent model and upregulation of skin-protective genes and downregulation of detrimental tissue remodeling genes by hypothermia may contribute to its beneficial effects. Rizzo et al. [86] applied moderate hypothermia in the range of 31–33°C for 4 h both immediately after burn injury and in a delayed fashion, beginning 2 h after thermal injury model in rats. Immediate hypothermia decreased burn depth progression at 6 h after injury, and this protective effect was sustained for at least 24 h. Increased expression of several skin-protective genes and decreased expression of tissue remodeling genes were discovered in the skin biopsy samples of rats subjected to immediate hypothermia.

9.4. Analgesia. Peripherally active opioids have anti-inflammatory effects and can modulate wound healing. Local opioid application is being used for pain reduction in patients with inflammatory lesions such as burns [87]. Opioid agonists can

attenuate the excitability of primary afferent neurons and block the release of proinflammatory neuropeptides from central and peripheral terminals [88]. The discovery that opioid receptors on sensory nerves are upregulated during subcutaneous inflammation prompted the search for endogenous ligands within inflamed tissue. Opioids can interfere at several different stages in the inflammatory process, both in somatic and visceral inflammations [89–91].

10. Conclusions

Burns are unique among acute injuries in the progressive nature of tissue necrosis and possible serious complications following the initial trauma, such as SIRS and severe metabolic imbalance. This intense instability in homeostasis may result in multiple organ failure and death.

Current treatment of burn shock includes prompt fluid resuscitation and early burn wound excision. However, aggressive crystalloid-based resuscitation strategies are also associated with inflammatory mediator dysfunction. Hence, the patient's care team must always be careful to restrict the supply of liquid to only what is necessary to rescue the hypovolemic shock. Early excision of burns plays an important role in attenuation of SIRS as it can decrease the release of inflammatory mediators and the bacterial colonization of wounds. Over the past twenty years, since the introduction of early excision and grafting in our burn unit, we have observed a reduction in the mortality rate from 12% to less than 5%.

Currently, alternative therapies are emerging that seek to modulate the complex systemic inflammation in burns. We support the discovery of new therapeutic options that can modulate the production of inflammatory cytokines which we believe will lead to improved treatment of burns. Inherently, the knowledge of the balance between pro- and anti-inflammatory cytokines is of fundamental importance and this also becomes a challenge to be overcome in the future. The aim of this paper is to comprehensively review current therapies available which can inhibit the inflammatory response to burns.

Abbreviations

SIRS:	Systemic inflammatory response syndrome
BSA:	Body surface area
mL:	Milliliter
kg:	Kilogram
LPC:	Lipid protein complex
TNF-α:	Tumor necrosis factor alpha
IL-1b:	Interleukin-1b
IL-6:	Interleukin-6
IL-8:	Interleukin-8
IL-10:	Interleukin-10
IFN-γ:	Interferon-gamma
EPC:	Endothelial progenitor cells
HC-FMRP-USP:	Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidadede São Paulo-Brazil;
Th1:	(T-helper-1) cytokines
APC:	Activated protein C
RBP:	Retinol binding protein
IR:	Insulin resistance
bFGF:	basic fibroblast growth factor
TGFα:	Transforming growth factor alpha
MB:	Methylene blue
VS:	Vasoplegic syndrome
NO:	Nitric oxide
cGMP:	Cyclic guanosine monophosphate
sGC:	Soluble guanylatecyclase
NOS:	Nitric oxide synthase
DNA:	Deoxyribonucleic acid
TGF-β1:	Transforming growth factor beta-1
DNAse:	Deoxyribonuclease
MSC:	Mesenchymal stem cell
Akt1-V:	Murine thymoma viral oncogene homolog 1
$^{\circ}$C:	Celsius degree
h:	Hour.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

The authors thank *BioMed Proofreading* for valuable edition by native English-speaking experts.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 985815, 10 pages
http://dx.doi.org/10.1155/2013/985815

Review Article

Curbing Inflammation through Endogenous Pathways: Focus on Melanocortin Peptides

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Received 22 February 2013; Revised 11 April 2013; Accepted 14 April 2013

Academic Editor: Christopher D. Buckley

The resolution of inflammation is now known to be an active process, armed with a multitude of mediators both lipid and protein in nature. Melanocortins are peptides endowed with considerable promise with their proresolution and anti-inflammatory effects in preclinical models of inflammatory disease, with tissue protective effects. These peptides and their targets are appealing because they can be seen as a natural way of inducing these effects as they harness endogenous pathways of control. Whereas most of the information generated about these mediators derives from several acute models of inflammation (such as zymosan induced peritonitis), there is some indication that these mediators may inhibit chronic inflammation by modulating cytokines, chemokines, and leukocyte apoptosis. In addition, proresolving mediators and their mimics have often been tested alongside therapeutic protocols, hence have been tested in settings more relevant to real life clinical scenarios. We provide here an overview on some of these mediators with a focus on melanocortin peptides and receptors, proposing that they may unveil new opportunities for innovative treatments of inflammatory arthritis.

1. Inflammation: Onset and Resolution

One novel approach to the area of inflammation, developed over the last twenty years, is the concept of resolution of inflammation. Current therapies suppress active processes of inflammation, for example, NSAIDs (nonsteroidal anti-inflammatory drugs) block cyclo-oxygenases, glucocorticoids inhibit generation of multiple cytokines, and biologics such as anti-TNFα and anti-CD20 therapies, target specific effectors or antigens. However, this may be only half the story. The story of inflammation begins with a tissue insult originating from an infection, trauma, or damage. The affected tissue secretes signals including autacoids, plasma-derived mediators such as kinins and complement factors, culminating with the now prominent cytokines and chemokines. There are multiple molecules that constitute a distress signal. This leads to an initial recruitment of neutrophils, (or eosinophils, upon parasite attack) which mop up any initial infection and call in macrophages, which are also inflammatory. Once neutrophils and macrophages have cleared the inflammation, the neutrophils undergo apoptosis, the macrophage changes its phenotype into a proresolving and tissue repair one, and then leaves and the tissue should return to its baseline uninflamed state [1]. However this return to baseline is not, as was once thought, characterised solely by absence of the inflammatory insult but it results also from a positive process with its own armamentarium of mediators that bring the tissue from an inflammatory state back into its normal resting state (Figure 1).

There are several processes of clearance of inflammation that lead to the return to the normal state (catabasis) [2]. Exclusion of the primary insult, for example, phagocytosis of invading bacteria, is foremost as this stops the synthesis of proinflammatory mediators. There is then the breakdown

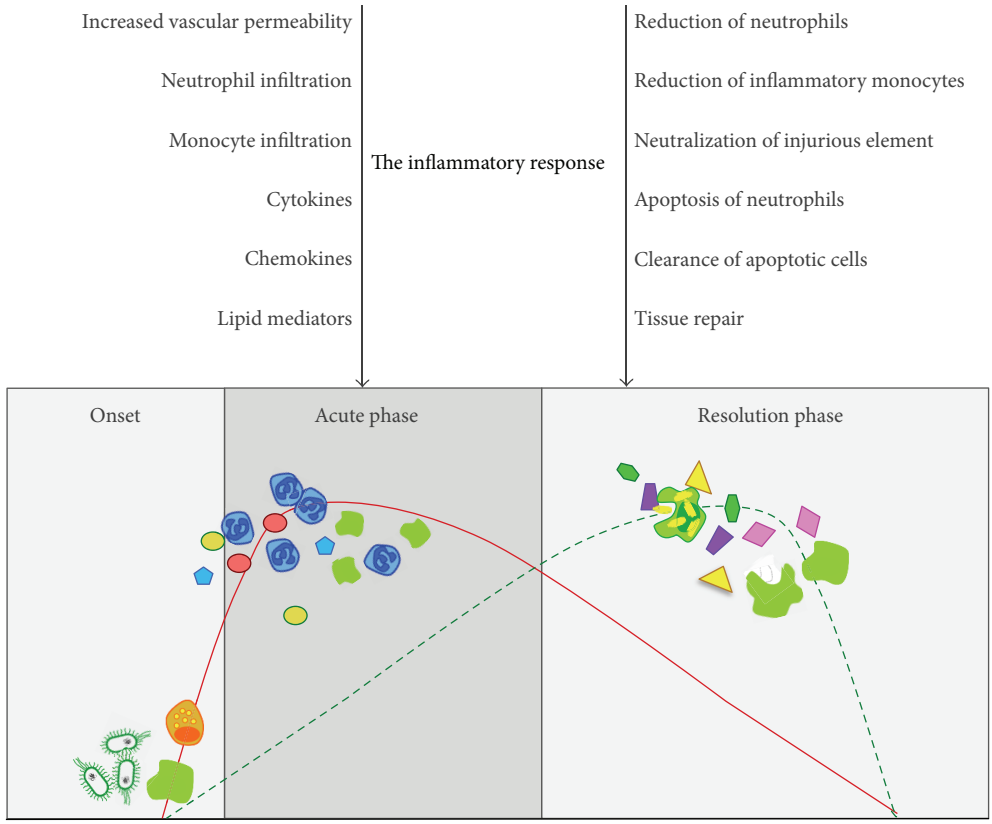

Figure 1: The inflammatory response. Stimuli such as tissue injury or microbial invasion trigger the release of chemical mediators (complement, cytokines, eicosanoids, and other autacoids) that activate the leukocyte recruitment (onset). Neutrophils are the first cell type to be recruited, and then peripheral blood monocytes also accumulate at the inflammatory site (acute phase). These monocytes will eventually differentiate into a more phagocytic phenotype helping to neutralize the injurious element and to clear the tissues off apoptotic neutrophils (resolution phase). This proresolving macrophage (and the involvement of stromal cells cannot be excluded here either) orchestrate resolution, by releasing and/or responding to proresolving mediators, some of which have been discussed in this review (see main text). Eventually, fully differentiated cells that have cleared the site by debris, dead cells, and bacteria will leave (via the lymphatic?) and the previously inflamed tissue or organ will regain its functionality, with return to homeostasis.

of the proinflammatory stimuli and also the cessation of production of these proinflammatory cytokines, chemokines, and other inflammatory mediators such as MMPs (matrix metalloproteinases) and proteolytic enzymes. This is the process that is targeted by most current therapy. Then there is the removal of the inflammatory cell infiltrate. This can be local cell death, usually by apoptosis followed by phagocytosis by macrophages (M2 phenotype, with anti-inflammatory remit) that then leave the site by lymphatic drainage [3]. Some of these macrophages themselves may die by apoptosis and be cleared by other resident cells. The crucial concept is that ingestion of the apoptotic neutrophils by macrophages (efferocytosis) would prevent the appearance of necrotic cells, which eventually will release their harmful content, therefore perpetuating the inflammatory response. In addition, this process is nonphlogistic; that is, it does not induce an inflammatory response [4]. Some cells might recirculate systemically and leave the site of inflammation [5]. The resolution phase of an acute inflammatory process can be defined in histological terms as the interval from maximum neutrophilic infiltration to the absence of neutrophilia [1].

There is now a host of mediators that are involved in the resolution phase of inflammation. Some of these are autacoids like adenosine, locally generated hormones like melanocortins and somatostatin, bioactive lipids like lipoxins, resolvins, protectins and maresins, proteins like heme oxygenase 1, annexin A1, galectins, and erythropoietin. Due to space limitation, we will discuss here a few examples of proresolving proteins and peptides in the melanocortin system.

2. The Melanocortin System as Archetypal for Proresolving Endogenous Mediators and Targets

In 1950, Philip Hench won the Nobel prize for treating patients with rheumatoid arthritis (RA) with cortisone [6, 7]. What is less well known is that he treated 6 patients with adrenocorticotrophic hormone (ACTH) with equally good results, as reported in that seminal paper. ACTH is the prototype of the melanocortins and its anti-inflammatory actions

have been confirmed and formed the basis for its use in the clinical management of inflammatory arthritides such as in the treatment of gout, where it is still used in the USA today. A placebo controlled trial of synacthen, a synthetic form of ACTH, in patients with RA showed an additional benefit which lasted three months after two injections on alternate days [8]. ACTH was evaluated in the treatment of gout patients, with relative contraindications to NSAIDS, ACTH was found to have good effect over and above that which would be expected from the release of endogenous cortisol alone [9]. The later discovery of the proopiomelanocortin system with a number of melanocortins and melanocortin receptors (MCR) has improved our understanding of the biological basis of these effects.

2.1. Melanocortin Receptors. The melanocortin system (Table 1) encompasses five melanocortin receptors, their ligands (agonists and antagonists), and the accessory proteins. The melanocortin receptors are a family of five small stimulatory G protein-coupled receptors, termed MC_1 to MC_5, initially identified as neuropeptide receptors in mice and humans in the early 1990s [21–27]. Each receptor has seven transmembrane domains, with an extracellular amino-terminus and short cytosolic carboxy-terminus. The melanocortin 2 receptor (MC_2) is the only one of the five which has been shown to require an accessory protein for translocation to the cell membrane [28]. However the presence of the accessory proteins (MRAP1 or MRAP2 (melanocortin receptor accessory protein)) may have an effect on the surface expression of the other melanocortin receptors and their ability to be activated by agonists [29, 30].

All melanocortin receptors signal via the cyclic AMP (cAMP) pathway, activating adenylate cyclase resulting in increased intracellular cyclic AMP [31–33]. Activation of certain MCRs has also been shown to mobilise calcium from intracellular stores [34–36] in certain cell types or conditions. For example, activation of MC_3 with alpha-MSH can result in increases in intracellular calcium in the presence of the protein kinase A inhibitor H-89 [31]. Similarly, if the cAMP pathway is blocked then MC_1 can signal via intracellular calcium mobilisation or the inositol trisphosphate pathway [37, 38]. MC_1 has been shown to affect the NFκB pathway by protecting IκBα from degradation leading to a downregulation of inflammatory cytokines and chemokines [33, 39].

In terms of distribution, melanocortin receptors are found in the brain and in peripheral tissues. It is notable that MC_1, MC_3, and MC_5 are expressed on multiple cells of the immune system suggesting a role in inflammation. Of note to the rheumatologist, MC_1 and MC_5 are present in human articular chondrocytes [40] and rheumatoid synovial fibroblasts. These cells are known to be part of the chronic immune response of rheumatoid arthritis and represent a source of effector cells for endogenous ligand.

2.2. Melanocortin Receptor Ligands. The ligands for the melanocortin receptors are derived from the proopiomelanocortin system. Proopiomelanocortin (POMC) is the precursor protein, from which prohormone convertases cleave the melanocortin stimulating hormones (MSH) alpha-, beta- and gamma-MSH and ACTH as well as the non-melanocortin peptides, beta-lipotropin, gamma-lipotropin, and beta-endorphin. Initially, POMC and its related components were thought to be restricted to the pituitary but now have been shown to have a wider distribution [41]. Table 2 summarizes the endogenous and synthetic melanocortin peptides.

MC_2 only responds to ACTH while the other melanocortin receptors respond to the melanocortin stimulating hormones to differing degrees [42]. MC_1 responds to alpha-MSH>ACTH>gamma-MSH, as does MC_5, whilst MC_3 responds to gamma-MSH=ACTH>alpha-MSH. As well as endogenous agonists, there are endogenous antagonists in both the mouse and human system [43]. These are known as agouti and agouti-related protein in the mouse and agouti signalling protein in the human. Other regulators in mice are mahogany, syndecan and the mahogunin ring finger 1 [44].

2.3. Anti-Inflammatory and Antiarthritic Actions of Melanocortin Peptides. The anti-inflammatory actions of alpha-MSH have been shown using *in vitro* studies on stable cell lines and human primary cells, as well as *in vivo* models of diseases such as rheumatoid arthritis, colitis or ischaemia reperfusion injury. Alpha-MSH was initially found to be an antipyretic, able to counteract the pyrogenic activities of IL6 and TNFα [45]. Manna and Aggarwal then showed that alpha-MSH suppressed proinflammatory cytokine production by monocytes in response to bacterial lipopolysaccharide, by inhibiting NFκB translocation to the nucleus [33]. Not only does alpha-MSH suppress proinflammatory cytokines, but also it can activate the production of anti-inflammatory cytokines such as IL10 from monocytes [46] and keratinocytes [47]. Alpha-MSH has been shown to be inhibitory in several inflammatory models. It is effective in experimental contact dermatitis and suppresses the sensitisation and elicitation phase of the immune response. Alpha-MSH induces hapten specific tolerance when given intravenously and this response is dependent on the induction of IL10 [48]. This finding has been taken forward in the nickel-induced contact eczema model in humans where a topical application of alpha-MSH reduced disease [49]. Alpha-MSH has been used in a model of cutaneous vasculitis induced by LPS and the peptide was able to reduce vascular damage and haemorrhage by downregulating cell adhesion molecules crucial for the extravasation of leukocytes to the site of inflammation [50]. Alpha-MSH has been topically applied to an airways model of allergy sensitised to ovalbumin, proallergic cytokines were reduced, and the anti-inflammatory action of alpha-MSH was dependent on IL10 [51].

Melanocortin agonists have been investigated in models of stroke encompassing mouse, rat, and gerbil models and also global and local ischaemic models. Gerbils, given ten minutes of global cerebral ischaemia by the occlusion of both carotid arteries, had reduced neuronal death, hippocampal damage and improved functional recovery if treated with an alpha-MSH derivative with a longer half-life (Nle^4, D-Phe^7 alpha-MSH, NDP-MSH) between three and nine hours after

Table 1: The melanocortin system.

	Melanocortin Receptors				
	MC_1	MC_2	MC_3	MC_4	MC_5
Endogenous agonists	αMSH=ACTH> βMSH>γMSH	ACTH	γMSH>ACTH =αMSH=βMSH	αMSH=ACTH> βMSH>γMSH	αMSH>ACTH= βMSH$\gg\gamma$MSH
Distribution	Skin Melanocytes Keratinocytes Endothelial cells Mucosal cells Adipocytes Chondrocytes Osteoblasts Macrophages Monocytes Dendritic cells Mast cells Neutrophils CD8+ T cells B Lymphocytes	Adrenal glands Adipocyte Osteoblasts Dendritic cell Chondrocyte	Hypothalamus Macrophages Monocytes Dendritic cells CD4+ T cells B lymphocytes	Hypothalamus Dendritic cells Osteoblasts	Exocrine glands Sebocytes Macrophages Dendritic cells Mast cells Chondrocyte CD4 T cells B lymphocytes NK cells
Signalling pathways	cAMP ERK1/ERK2	cAMP	cAMP Intracellular [Ca^{2+}]	cAMP	cAMP Intracellular [Ca^{2+}] Jak/STAT
Biological functions	Skin pigmentation Inflammation Wound healing	Steroidogenesis	Energy homeostasis Inflammation	Energy homeostasis Food intake Erectile function	Exocrine glands function, Inflammation Defensive behaviour
Role in disease/Potential use	Skin cancer Inflammation Alopecia areata (?) Vitiligo	Familial glucocorticoid deficiency	Inflammation Gouty arthritis Obesity Tuberculosis (?)	Obesity Cachexia Sexual dysfunction	Seborrheic dermatitis Acne vulgaris Inflammation

insult. Interestingly MC_4 blockade abrogated the effects of the NDP-MSH suggesting the activity of MC4R in this process [52]. In human studies, alpha-MSH levels in the plasma have been used as a biomarker for predicting functional recovery from stroke [53].

Alpha-MSH and its analogues have also been used in preclinical models of renal and lung injury, secondary to sepsis or other forms of injury. It has been shown in multiple models to ameliorate injury with improvements in histology and plasma creatinine compared to controls. AP214, a nonselective melanocortin agonist derived from alpha-MSH, has been used in a sepsis-induced kidney injury model. Treatment with AP214 was delayed until six hours after the onset of sepsis and still reduced damage to the kidney as monitored by histological score, tubular damage, and serum creatinine; these effects were associated also with an improved liver function [16]. AP214 also reduced serum TNFα and IL10 and showed evidence of reduced NFκB activation. There was also an improvement in survival rate in both lethal sepsis groups (improved from 0% survival to 10% survival) and sublethal sepsis groups (an improvement from 40% survival to 70% survival). This has been reflected in other studies of kidney injury models where alpha-MSH was given up to 6 hours after injury, observing increased recovery and protection against renal injury [54].

Alpha-MSH also ameliorates liver inflammation—as assessed following endotoxin induced inflammation in mice-even if given 30 minutes after onset, with decreased neutrophils infiltration and also decreased gene expression of chemotactic cytokines such as MCP1 (monocyte chemoattractant protein) and IL8 as well as TNFα [55]. Severe tissue injury in the lung can lead to acute respiratory distress syndrome as can renal ischaemic reperfusion injury with similar pathways activated in both organs. Alpha-MSH can inhibit lung oedema decrease injury score and leukocyte infiltrate as well as decreasing serum creatinine and improving histology score in the kidney. Gene expression of TNFα and ICAM1 (intercellular adhesion molecule) is reduced in the lung after treatment with alpha-MSH. Alpha-MSH also prevented the degradation of IκB, phosphorylation of p38 mitogen activated protein kinase and decreased AP1 binding suggesting that alpha-MSH can operate through various pathways to modulate the inflammatory response, rather than just triggering one method of dampening inflammation [56].

Melanocortin agonists have been used in the treatment of various experimental arthritis models. AP214 is a peptide

Table 2: State of the art for the development of melanocortin agonists.

Compound	Classification	Activity	Effects	References
αMSH	Endogenous	Pan agonist	Anti-inflammatory Skin pigmentation	[10]
βMSH	Endogenous	Pan agonist		
γMSH	Endogenous	Pan agonist with increased MC_3 selectivity	Anti-inflammatory	[11]
Agouti related peptide	Endogenous	Antagonist, MC_3, MC_4	Skin pigmentation	
Agouti signalling protein	Endogenous	Antagonist, MC_1, MC_3, MC_4	Skin pigmentation	
D-Trp^8-γMSH	Synthetic peptide	Agonist with increased MC_3 selectivity	Anti-inflammatory (arthritis)	[12]
NDP-αMSH (MT-I)	Synthetic peptide	Pan agonist	Anti-inflammatory	[10]
MT-II	Synthetic peptide	Pan agonist	Anti-inflammatory	[13]
KPV	Synthetic peptide	MC_1 agonist	Anti-inflammatory	[10]
KPT	Synthetic peptide	Pan agonist	Anti-inflammatory	[10]
(CKPV)2	Synthetic peptide	Pan agonist	Anti-inflammatory	[14]
GKPV	Synthetic peptide on beads	Pan agonist	Anti-inflammatory (melanoma)	[15]
AP214	Synthetic peptide	Pan agonist	Anti-inflammatory (sepsis and arthritis)	Action Pharma A/S [16, 17]
HP228	Synthetic peptide	Pan agonist	Protective in acute models of inflammation and organ damage	[18]
BMS470539	Small molecule	Agonist MC_1	Inhibits LPS response	[19]
ME10501	Small molecule	High affinity mMC_1, hMC_4	Neuroprotective	[20]
Bremelanotide	Small molecule	Agonist MC_1 and MC_4	Prevents organ dysfunction	Palatin Technologies
SHU-9119	Synthetic peptide	Antagonist at MC_3 and MC_4, agonist for MC_1 and MC_5	Experimental tool	
Afamelanotide	Synthetic peptide	Pan agonist	Vitiligo, acne vulgaris, erythropoietic protoporphyria, solar urticaria	Clinuvel Pharmaceuticals
RM-493	Synthetic peptide	Agonist MC_4	Obesity	Rhythm Pharmaceuticals, Inc
Czen001, 002	Synthetic peptide	Agonist	Anti-infective Anti-inflammatory	MSH Pharma

pan-agonist which has been shown to reduce the disease score in a mouse model of arthritis and induce proresolving properties (increased phagocytosis) in macrophages [17]. Carrier technology has been applied to alpha- and gamma-MSH and used in the CIA (collagen induced arthritis) and urate peritonitis models showing effective amelioration of inflammatory parameters of the two experimental diseases [11]. This approach aims to facilitate the targeting to inflammatory sites of unstable peptides such as melanocortins, by fusion with the latency-associated peptide (LAP) of TGFβ1 through a cleavable matrix metalloproteinase linker [57].

Alpha-MSH has been used to treat adjuvant arthritis in rats with an increase in body weight, reduction of the arthritis score, and erosions [58]. POMC gene therapy has been used to treat adjuvant arthritis in rats with a reduction in paw swelling after adjuvant injection as well as thermal hypersensitivity [59]. Melanocortins have also been studied in models of gouty arthritis. Alpha-MSH and a small peptide derivative (CKPV-2) have been shown to inhibit the ability of monocytes to produce neutrophils chemoattractants and activating compounds in response to urate crystals [60]. The melanocortin peptide $ACTH_{4-10}$ has been shown to reduce neutrophil accumulation in an *in vivo* model of crystal-induced peritonitis and to inhibit *in vitro* macrophage activation with reduced chemokine KC release [61]. By using the mixed MC_3/MC_4 antagonist SHU9119, this study identified MC_3 as being responsible for these actions, since peritoneal macrophages do not express MC_4. Also the agonist

melanotan II, a stable pan-agonist at all receptors, gave similar results as the alpha-MSH derivative [61, 62]. In the same system, two MC_3 agonists MT-II and gamma-MSH also inhibited neutrophil accumulation and release of cytokine and chemokines from macrophage. Furthermore MC_3 is expressed in the C57BL6 mouse and Sprague Dawley rat peritoneal macrophages, as determined by Western blot. ACTH reduced joint size and inhibited neutrophils accumulation in rat knee joints injected with urate crystals. SHU9119 abrogated the effectiveness of ACTH in this model while gamma-MSH showed similar protective qualities [63]. Further evidence suggesting that MC_3 is important in this model came with the efficacy of nonselective and selective MC_3 agonists in the amelioration of urate crystal-induced peritonitis in a mouse colony bearing a nonfunctional MC_1. This was further supported by presence of MC_3 protein in mouse peritoneal macrophages [64]. [D-Trp^8]gamma-MSH (a gamma-MSH derivative with preference for activating mouse MC_3) afforded protection when used for the treatment of rat gout arthritis or urate peritonitis, and but not when used in MC_3 deficient mice (only the urate peritonitis model has been tested), again suggesting a role for MC_3 in controlling the inflammation produced by urate crystals [65]. The same compound has been shown to be efficacious in murine peritonitis despite a nonfunctional MC_1, again guiding us to believe that MC_3 might be more relevant in this mouse model of gout [66]. Overall these experiments show the efficacy of ACTH and its derivatives, natural and synthetic, in the treatment of mouse and rat models of gout and suggest that MC_3 is the receptor mediating these effects.

2.4. Melanocortins in Human Arthritis. Little is known about the effects of melanocortins on human arthritis other than the effects of ACTH in rheumatoid arthritis and gout which have been known about since the 1950s [6, 7, 67–72]. The clinical efficacy of ACTH in gouty arthritis was reevaluated in the 1990s [9] and this retrospective study confirmed an efficacy over and beyond what one would expect from the release of endogenous cortisol. In the United States, ACTH is part of the clinical armamentarium for gout, especially for the treatment of patients with contraindications to NSAIDs. Catania et al. discovered elevated levels of alpha-MSH in the synovial fluid of rheumatoid arthritis and juvenile chronic arthritis patients compared to those with osteoarthritis (OA). Using paired samples, these authors also showed that the levels of alpha-MSH were elevated in synovial fluid as compared to serum. The concentrations of alpha-MSH were proportional to the degree of inflammation [73]. Bohm's and Grassel's groups have reported presence of melanocortin receptors 1 and 5 in human chondrocytes and have proposed a role for the melanocortins in the osteoarticular system [74]. Yoon et al. showed a reduction in MMP13 production and p38 kinase phosphorylation when human chondrosarcoma cells were pretreated with alpha-MSH and then stimulated with TNFα. This was independent of ERK and JNK kinases but reliant on p38 kinases and NFκB [75]. Addition of alpha-MSH to TNFα-activated human chondrocytes reduced production of proinflammatory cytokines and increased the release of the anti-inflammatory cytokine IL10 [76].

2.5. Would New Therapeutics Emerge from Research on Melanocortins? We conclude this overview by highlighting the therapeutic potential that the area of the resolution of inflammation may retain. For space limitation, we focus on the melanocortin research only, though it is clear that other effectors of resolution and their targets would also be endowed with promising opportunities. Only the future will tell if new therapeutics will indeed be developed out of this research effort.

Given the data from preclinical models and the success of alpha-MSH and its derivatives as well as *de novo* agonists, melanocortin ligands have been taken forward into clinical trials for further investigation in humans. The minimum peptide sequence from alpha-MSH that can activate MC receptors is a tri-peptide (KPV). However, although active, it has a very short half-life and much work has been based on modifying alpha-MSH and its derivatives to extend their duration of action. There is much effort on melanocortin peptides, with the aim of producing a preparation, that is, easy to deliver, specific to its target tissue, more selective, and with a longer half-life.

Possible side effects of melanocortin receptor stimulating drugs are skin pigmentation and increased risk of melanoma due to activation of MC_1, hypertension, and behavioural disturbances due to activation of MC_3, MC_4, and MC_5. Pan-agonists may activate the yawning and stretching reflexes stimulated by MC_4. An important aspect to consider for small molecules targeting MC_3 would be their inability to cross the blood brain barrier, thus preventing unwanted actions on food intake and central control of blood pressure [77].

Despite these potential side effects, melanocortin-based therapeutics are generally safe and well tolerated by patients. In addition, ACTH (which activates all melanocortin receptors, as discussed above) is currently used for the treatment of gout, but it displays efficacy also for proteinuric nephropathies [78], exacerbations of multiple sclerosis [79], and several rheumatic disorders [8, 9, 72, 80], indicating that targeting the melanocortin system might be a genuinely valid therapeutic approach. However, the paucity on appropriate randomized, controlled, double-blind trials to evaluate the efficacy of this drug has limited its use as well as development of melanocortin-based therapies.

The perception may be changing right now. In fact, we are experiencing a renaissance of the melanocortin field as many drugs are (or have been) subjected to clinical trials to treat a variety of conditions (http://clinicaltrials.gov). For example, the drug RM-493, a selective MC_4 peptide agonist from Rhythm Pharmaceuticals, Inc. is currently on a phase-II trial to evaluate its antiobesity effects. The drug bremelanotide (Palatin Technologies), which activates MC_1 and MC_4 receptors, is currently under investigation for female sexual arousal disorder, although the effects on blood pressure have questioned the risk/benefit ratio of bremelanotide for this indication. Action Pharma A/S completed a phase-II clinical trial using the compound AP214 (described earlier in this

review) to assess the prevention of kidney injury in patients undergoing cardiac surgery. In addition, the FDA Office of Orphan Products Development is running a trial to assess the effects of alpha-MSH on acute renal failure. A third study (phase II, completed) focused on nephropathies is conducted by Radboud University, in which they aimed to evaluate the effects of a synthetic ACTH on idiopathic membranous nephropathy. Diabetic nephropathy is another indication under evaluation in a phase IV clinical trial conducted by Southeast Renal Research Institute. ACTH is also being evaluated for the treatment of systemic lupus erythematosus and multiple sclerosis (Questcor Pharmaceuticals, Inc., phase IV and I, resp.). The peptide afamelanotide, an alpha-MSH derivative with increased stability and potency developed by Clinuvel Pharmaceuticals Limited, is currently under investigation for the treatment of erythropoietic protoporphyria (phase-III, recruiting), solar urticaria (phase II, completed), vitiligo (phase I), and for the treatment of acne vulgaris due to its anti-inflammatory properties (phase II, completed). Of note, companies such as MSH Pharma, Inc. include in their pipeline melanocortin drugs to treat conditions such as rheumatoid arthritis and inflammatory bowel disease, highlighting that there is a renewed current interest in developing melanocortin-based therapies for chronic inflammatory diseases and that melanocortin drugs are ready for translation.

Conflict of Interests

The authors have no conflicts of interest.

Acknowledgments

This work of the authors mentioned in this review was supported by the Arthritis Research UK (studentship 18551), the Wellcome Trust (project Grant 083551) and, in part, the William Harvey Research Foundation. Mauro Perretti and Costantino Pitzalis share senior authorship.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 780502, 9 pages
http://dx.doi.org/10.1155/2013/780502

Research Article

Evolution of the Macrophage CD163 Phenotype and Cytokine Profiles in a Human Model of Resolving Inflammation

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Received 26 February 2013; Accepted 7 April 2013

Academic Editor: Christopher D. Buckley

Cantharidin skin blisters were examined over two days to model the acute and resolving phases of inflammation in human skin. Four blisters were created by topical administration of cantharidin (0.1% v/v) to the forearm of healthy volunteers, with IRB approval. Duplicate skin blisters were aspirated at 16 and 40 hours to model the proinflammatory and resolving phases, respectively. There was a significant increase in leukocyte infiltrate at 40 h with appearance of a "resolving macrophage" phenotype $CD14^+CD163^+$ by flow cytometry. Neutrophils acquired apoptotic markers at 40 h and were observed to be phagocytosed by macrophagic "Reiter's" cells. Multiplex cytokine analysis demonstrated that monocyte chemoattractant protein (MCP-1/CCL2), interleukin- (IL-) 6, IL-8/CXCL8, macrophage inflammatory protein (MIP1α/CCL3), MIP-1β/CCL4, tumor necrosis factor- (TNF-) α, and eotaxin (CCL11) were all significantly upregulated at 16 h compared with 40 h. In contrast, immunoregulatory transforming growth factor- (TGF-) β, macrophage-derived chemokine (MDC/CCL22), and interferon-inducible protein (IP-10/CXCL10) were significantly elevated at 40 h. Our results demonstrate that the phases of inflammation and resolution can be discriminated in a two-day model of dermal wound healing. This confirms and extends our understanding of wound repair in humans and provides a powerful research tool for use in clinical settings and to track the molecular benefits of therapeutic intervention.

1. Introduction

The wound healing process is balanced between an early cytodestructive inflammatory phase and a subsequent resolving phase supporting tissue regeneration [1, 2]. Cells of the monocyte/macrophage lineage have been recognized since the 1970s to participate actively in both of these phases [3]. A key switching point is the conversion of the proinflammatory monocyte into a macrophage phenotype capable of dampening the inflammatory response and moulding fibrosis [4, 5]. The prototypic marker of this conversion is CD163 (the haemoglobin scavenger receptor), first recognised as the "resolving macrophage marker" in humans and as the ED2 antigen in rats [6, 7].

CD163 plays an everyday role in neutralising pro-oxidant free heme released during hemolysis in bruising or tissue injury. In a rat model of lung injury, it was expressed during inflammatory resolution at which macrophages engulfed apoptotic neutrophils [8]. In a model of gout involving monosodium urate crystal phagocytosis, the $CD163^+$ phenotype evolves at the stage when monocytes/macrophages switch production from proinflammatory TNFα/IL-1/IL-6 cytokines to anti-inflammatory TGF-β [9]. In atherosclerotic plaques, $CD163^+$ macrophages dampen oxidative injury due to intraplaque hemorrhage [10, 11]. The $CD163^+$ phenotype is therefore a hallmark of the wound healing macrophage [12].

Despite a wealth of research in animal models, a molecular dissection of wound repair in humans and the role played by macrophage differentiation have been difficult to achieve due to lack of a convenient model.

The early inflammatory phase of wound repair is characterised by a predominantly granulocytic wave of leukocyte recruitment governed by neutrophil chemoattractants like Gro-α (CXCL1) and IL-8 (CXCL8) [13]. However, monocytes also participate in the first wave with IL-1, IL-6, TNFα, and MCP-1/CCL2 as prominent chemoattractants [13–16]. There

remains some debate as to the importance of MIP-1α/CCL3 and MIP-1β/CCL4 in humans, but these mediators recruit leukocytes to wounds in animals [14, 17].

The blistering agent cantharidin has been in clinical use since the 1970s and is used as a topical treatment for molluscum contagiosum and warts. It is a protein phosphatase 1 and 2 alpha inhibitor [18]. When applied to skin it causes acantholysis and blister formation [19]. No serious adverse reaction for topical use of cantharidin has been reported in the literature [20]. The experimental use of cantharidin, as a model to study leukocyte trafficking, involves topical application of cantharidin at one seventh the clinical dose to the forearm, causing a blister of median volume 0.5 mL [21]. Blister fluid sampled between 16 hours and 24 hours exhibits the hallmarks of acute inflammation, with infiltration of inflammatory leukocytes and accretion of inflammatory cytokines, such as IL-8 and TNFα. In mice, an analogous model of inflammation has been developed using cantharidin in an ear swelling model [22]. We have further refined this technique to allow comprehensive analysis of the surface phenotype on blister emigrated leukocytes by flow cytometry [23].

Here, we have investigated whether cantharidin skin blisters can be extended into a second day to model the resolving phase of wound repair in human skin. We have compared chemokine and cytokine profiles in blister fluid at 16 hours versus 40 hours using multiplex technology and monitored conversion of the monocyte/macrophage lineage into the wound healing CD163 phenotype by flow cytometry.

2. Methods

2.1. Reagents. Cantharidin (Cantharone) was purchased from Dormer Laboratories Inc. (Rexdale, ON, Canada). Anti-CD163-FITC was purchased from Bachem (Merseyside, UK). Anti-CD14-ECD, control IgG-ECD, and control IgG-FITC were purchased from Beckman Coulter (High Wycombe, UK). Anti-CD16-FITC was purchased from Serotec (Kidlington, UK).

2.2. Human Subjects. Ten healthy human volunteers were enrolled into this study with informed consent. The study protocol was approved by the Hammersmith Hospitals Research Ethics Committee. One adverse event (hyperpigmentation that persisted after the blister had healed) was reported to the Research Ethics Committee, but the hyperpigmentation resolved eventually and enrolment was allowed to continue using a revised consent form. All human investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.3. Cantharidin-Induced Skin Blisters. A total of four skin blisters were created on the volar aspect of the forearm in each subject by topical application of Cantharone (Dormer Laboratories) at a concentration of 0.1% in acetone as described [23]. Two skin blisters were randomly assigned to the 16-hour timepoint and two skin blisters to the 40-hour timepoint. Blister fluid was collected into siliconized microcentrifuge tubes (Sigma Aldrich, Poole, UK) and stored on ice prior to cell counting and flow cytometric analysis. Total viable cell counts were determined using Trypan Blue stain, followed by counting in a hemocytometer. Differential cell counts were performed on a subset of 7 subjects using Kimura's stain, with counting in a hemocytometer. Blister supernatants were collected after microcentrifugation and stored for analysis of chemokines and cytokines at −70°C.

2.4. Flow Cytometric Analysis. Flow cytometric analysis of leukocytes was performed immediately after aspiration of blisters and microcentrifugation of samples, with all incubation and washing steps carried out in ice-cold PBS. CD163 expression on monocyte/macrophages was carried out in the fluorescent-(FL-)1 channel on cells gated with anti-CD14 antibody in the FL-3 channel. CD163 expression in whole blood was determined using the Immunolyse whole blood lysing technique (Beckman Coulter, Luton, UK.) as previously described. Apoptosis on the gated neutrophil population was carried out by two methods: measuring loss of expression of CD16 in the FL-1 channel or using the Annexin V-FITC Apoptosis Assay in the FL-1 and FL-2 channels as per manufacturer's recommendations (BD-Pharmingen, San Diego, CA).

2.5. Multiplex Cytokine/Chemokine Analysis. The human cytokine multiplex-25 bead array kit was purchased from Biosource International (Camarillo, CA). The following cytokines/chemokines were screened: eotaxin (CCL11), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-(IL-)1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-10, IL-12(p40/p70), IL-13, IL-15, IL-17, IL-1 receptor antagonist (IL-1RA), interferon-(IFN-)α, IFN-γ, interferon-γ inducing protein 10 kDa (IP-10/ CXCL10), monocyte chemotactic protein (MCP-1/CCL2), macrophage inflammatory protein (MIP-1α/CCL3), MIP-1β/CCL4, regulated upon activation normal T cell expressed and secreted (RANTES/CCL5), monokine induced by γ-interferon (MIG/CXCL9), and tumor necrosis factor-(TNF-)α. Standard curves for each cytokine (in duplicate) were generated by using the reference cytokine concentrations supplied in this kit. Blister samples were diluted 2-fold in appropriate assay diluent. The assay was performed in a 96-well filter plate, using all the assay components provided in the kit. All incubation steps were performed at room temperature and in the dark to protect the beads from light. Samples were analysed using the Luminex 100 IS Multiplex Bio-Assay Analyzer (Bio-Rad Laboratories, Hercules, CA).

2.6. Enzyme-Linked Immunosorbent Assays. Transforming growth factor-(TGF-) β, macrophage-derived chemokine (MDC/CCL22), IL-8/CXCL8, and MCP-1/CCL2 protein levels in blister fluid were measured in triplicate by capture ELISA (R&D Systems, Abingdon, UK) according to the manufacturer's recommendations. Cytokine/chemokine levels were expressed as mean concentration (pg/mL) ± SE.

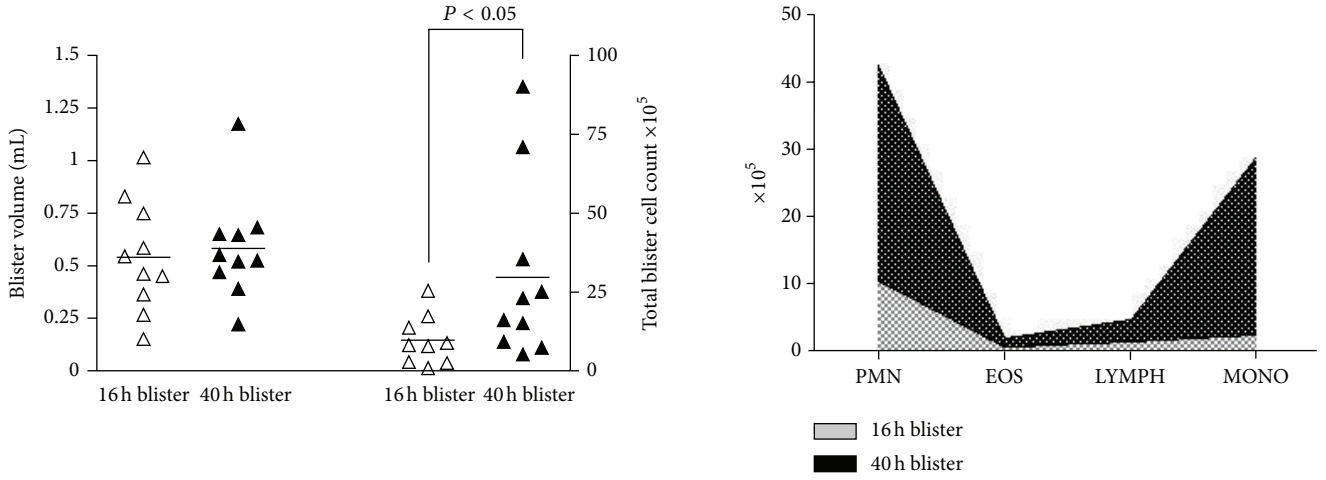

Figure 1: Leukocyte infiltration into cantharidin-induced skin blisters. Quadruplicate skin blisters were established by topical application of cantharidin (0.1%) to the forearm of 10 healthy individuals. Blister fluid was collected from duplicate blisters at 16 h and 40 h, respectively, and analyzed as follows (a) Fluid volume (mL) and cellularity ($\times 10^5$). Each point represent the average from 2 skin blisters; horizontal lines represents the population mean. (b) Differential leukocyte subpopulations. PMN: polymorphonuclear phagocytes; EOS: eosinophils; LYMPH: lymphocytes; MONO: monocytes/macrophages.

2.7. Protein Membrane Array Assay. The RayBio cytokine membrane assay kit 5.1 allowed detection of 79 different human cytokines and chemokines from a single 1 mL fluid sample. Representative 16-and 40-hour blister supernatant samples were thawed slowly at 4°C and diluted 1 : 10 before performing the membrane array assay according to the manufacturer's instructions (RayBiotech Inc., Norcross, GA).

2.8. Statistical Analysis. Comparisons between groups were analysed using an unpaired Student's *t*-test. Statistical analysis was carried out using Graphpad Prism software (GraphPad Software Inc., La Jolla, CA), and significance was assumed at $P < 0.05$.

3. Results

3.1. Analysis of Leukocyte Infiltrates into Skin Blisters at 16 Hours and 40 Hours. There was a statistically significant increase in leukocytes infiltrating into skin blisters at 40 hours compared to 16 hours ($P < 0.05$; Figure 1(a)). However, the volume of blister fluid did not alter significantly (Figure 1(a)). Blister cellularity was not correlated with blister volume ($r^2 = 0.03$; $P = 0.54$). Analyzing the leukocyte subpopulations within blister fluid revealed a marked increase in the number of neutrophils and monocytes/macrophages per blister present at the 40-hour timepoint (Figure 1(b)). The proportion of neutrophils, monocytes/macrophages, lymphocytes, and eosinophils present in blister fluid is illustrated in Table 1.

The morphological appearance of neutrophils by light microcpopy altered over time, consistent with apoptosis. Vacuolated cytoplasm, condensation, and cell membrane degradation were observed at 40 h (Figure 2(b)). Also detected in cytospin, preparations of blister fluid at 40 hours were large macrophages that had engulfed apoptotic neutrophils ("Reiter's" cells).

Table 1: Proportion of leukocyte subsets in skin blisters.

	Neutrophils	Eosinophils	Lymphocytes	Monocytes
16 h blister	68.9%	6.6%	7.7%	16.8%
40 h blister	56.5%	6.0%	7.9%	29.7%

3.2. Flow Cytometric Determination of Neutrophil Phenotype. Forward and side-scatter profiles of the granulocyte gated population in blister fluid revealed a distinct smaller, less granular sub-population at 40 hours (Figure 3(a)). By placing a gate over this new cell population, these were shown by Annexin V and Propidium Iodide staining to contain apoptotic and necrotic cells (Figure 3(a)). These cells also exhibited diminished expression of CD16, a characteristic of neutrophil apoptosis [24]. Whereas $CD16^{low}$ cells comprised 10.05% ± 9.72 (mean ± S.D.) of the population at 16 hours, this rose to 37.6% ± 28.3 at 40 hours ($P < 0.05$) (Figure 3(b)). Hence, flow cytometric data for apoptosis markers supported the observations of light microscopy showing an increase in apoptotic neutrophils at 40 hours in the blister transudate.

The purity of gated leukocyte sub-populations was verified by CD16/VLA-4 double-staining in the granulocyte and mononuclear cell gates. The percentage of $CD16^{-}VLA\text{-}4^{+}$ (monocytic cells) cells contaminating the granulocyte gate was 2.2% ± 0.7 (mean ± SEM, $n = 9$), and likewise the percentage of $CD16^{+}VLA4^{-}$ (neutrophilic) cells in the mononuclear gate was 5.0% ± 0.8, confirming that the gating strategy based on forward and side-scatter profiles combined with $CD14^{+}$ marker was specific enough to discriminate

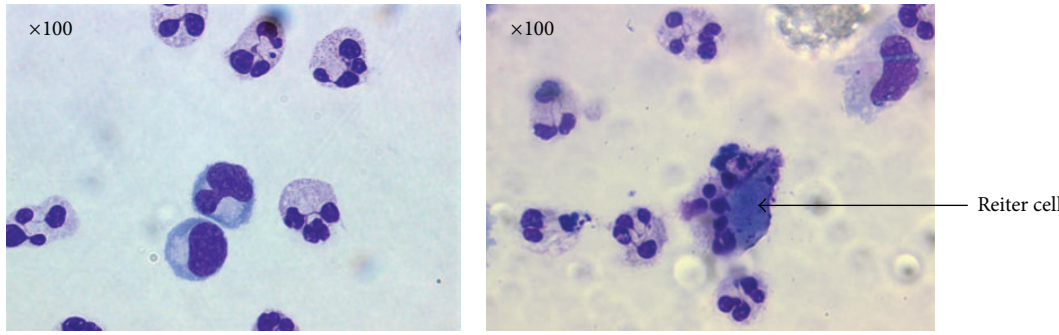

Figure 2: Photomicrographs of blister fluid at (a) 16 hours and (b) 40 hours. Granulocytes reveal morphological changes characteristic of apoptosis at 40 hours, including vacuolation of cytoplasm, size shrinkage, and engulfment by phagocytic "Reiter's" cells (arrow). Magnification = 100x.

between neutrophil and monocyte/macrophage lineage populations.

3.3. Flow Cytometric Determination of Monocyte/Macrophage Phenotype. CD163 is a monocyte-macrophage lineage marker expressed by alternatively-activated macrophages during the resolving phase of inflammation [7, 25]. The monocyte/macrophage population at 40 hours shows clear evidence for differentiation into an alternatively activated endpoint, with a significant increase ($P < 0.01$) in the proportion of $CD14^+CD163^+$ double positive cells (47.6%±7.6 at 40 h (mean ± S.D.) compared to 3.4%±1.1 at 16 h and 4.0%± 1.1 within the circulation). This adds to the evidence that by 40 hours the cellular infiltrate within the blister reflects a resolving macrophage phenotype.

3.4. Chemokine and Cytokine Expression in Blister Supernatant. To examine the soluble inflammatory mediators within blister supernatants, a cytokine array was chosen as an initial screening step prior to quantitative analysis by ELISA. The array could detect up to 79 different cytokines using only 1 mL of blister sample fluid. Blister supernatant from a randomly selected individual was diluted 1 : 10 and run on the array. The results pointed towards a changing inflammatory status within the blister over time (data not shown). The 16 h blister supernatant was strongly positive for proinflammatory cytokines IL-8/CXCL8 and MCP-1/CCL2, but by 40 h these cytokines had decreased. A weak signal for MDC/CCL22 was detected at 40 hours and submitted for confirmatory testing by ELISA. ELISA confirmed the observations of the protein array (Figures 4(a)–4(c)), with IL-8/CXCL8 and MCP-1/CCL2 significantly elevated at 16 hours, but MDC/CCL22 exhibiting an opposite profile (i.e., higher at 40 hours).

Next, blister fluid from the complete study panel ($n = 10$) was analysed by multiplex bead array for the presence of 25 human cytokines, chemokines, and growth factors. The results of this analysis confirmed the previous observations for IL-8 and MCP-1 by protein array and ELISA, showing a statistically significant increase at 16 h compared to 40 h (Figures 5(a) and 5(b); $P < 0.05$). Similar patterns were also observed for five other pro-inflammatory mediators, namely, TNFα, IL-6, MIP-1α, MIP-1β, and eotaxin (Figures 5(c)–5(g); all statistically significant $P < 0.03$, except for IL-6 trend, $P = 0.235$). The opposite pattern was observed for IP-10, which was elevated at 40 h compared to 16 h (Figure 5(h); statistical trend $P = 0.073$). There were no statistically significant changes or trends in expression for the following cytokines/chemokines/growth factors: IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-7, IL-10, IL-12(p40/p70), IL-13, IL-15, IL-17, GM-CSF, IFN-α, IFN-γ, IP-10/CXCL10, MIG/CXCL9, and RANTES/CCL5. Finally, the important wound healing cytokine TGF-β, which was not represented on the bead array, was shown by ELISA to have the same expression pattern as MDC/CCL22 and IP-10/CXCL10 (i.e., elevated at 40 hours compared to 16 h hours) (Figure 4(d); $P = 0.004$).

4. Discussion

The present study establishes cantharidin skin blisters as a tool for tracking the two main phases of wound healing in humans. By extending blisters into a second day and by analyzing blister infiltrates using flow cytometry and multiplex cytokine arrays, we were able to detect the following hallmarks in the transition from a pro-inflammatory phase at 16 h to a resolving state at 40 h: (1) a switch from proinflammatory mediators (IL-6, IL-8/CXCL8, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, TNFα, and eotaxin/CCL11) to immunoregulatory chemokines and growth factors (TGF-β, MDC/CCL22, and IP-10/CXCL10), (2) transition of $CD14^+CD163^-$ monocytes to a resolving macrophage $CD14^+CD163^+$ phenotype, and (3) acquisition of apoptosis markers by neutrophils and evidence for their phagocytosis by Reiter's cells.

The cantharidin blister model has enabled a molecular dissection of the wound healing process in humans, which has confirmed and extended our existing understanding of dermal wound repair in humans. Our data confirms the importance of IL-6, IL-8/CXCL8, MCP-1/CCL2, MIP-1α, MIP-1β, and TNFα in the early inflammatory phase [13, 14, 16, 26, 27]. New insights were gained into eosinophil recruitment into skin, with eotaxin exhibiting an early expression profile similar to MCP-1/CCL2 and IL-8/CXCL8. This is consistent with a prominent role for this cell type in the wound repair

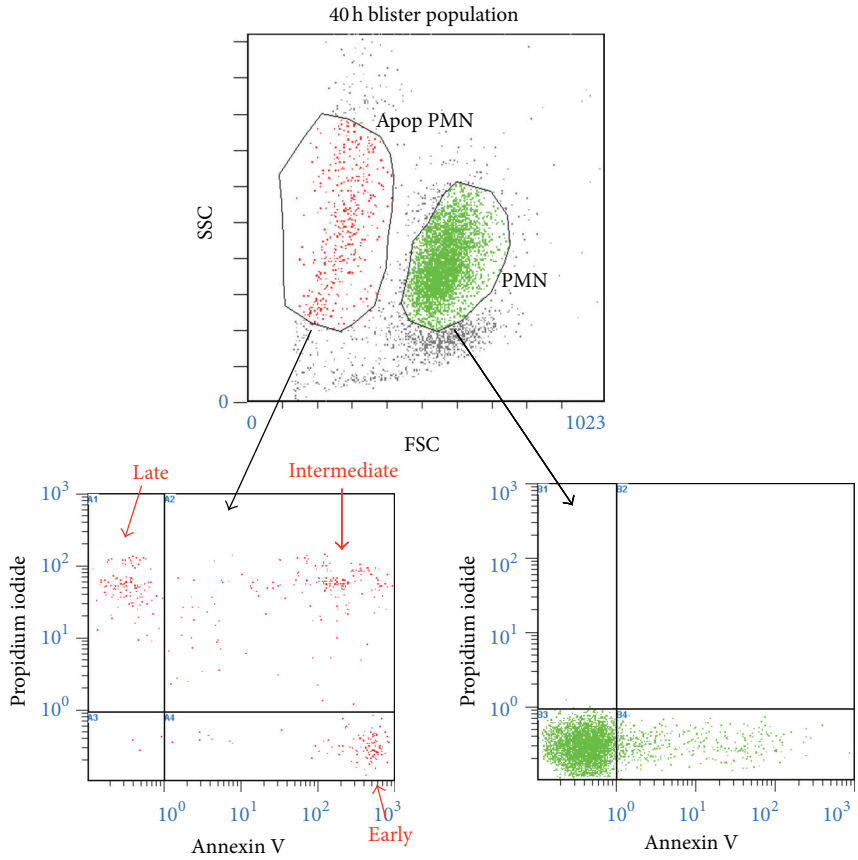

Figure 3: Flow cytometric analysis of PMNs in blister fluid. (a) Forward and side-scatter analyses reveal a subpopulation of smaller, less granular PMN. Gating on this subpopulation demonstrates expression of apoptosis markers, Annexin V, and Propidium Iodide (PI). Annexin V^+ cells are considered at an early stage of apoptosis, Annexin V^+/PI^+ cells are at an intermediate stage, and Annexin V^-/PI^+ cells at a late stage of apoptosis. No expression of apoptosis markers is seen in the viable cell gate. (b) PMNs at 40 hours demonstrate an increased proportion of cells exhibiting a $CD16^{low}$ phenotype, also characteristic of apoptotic cells.

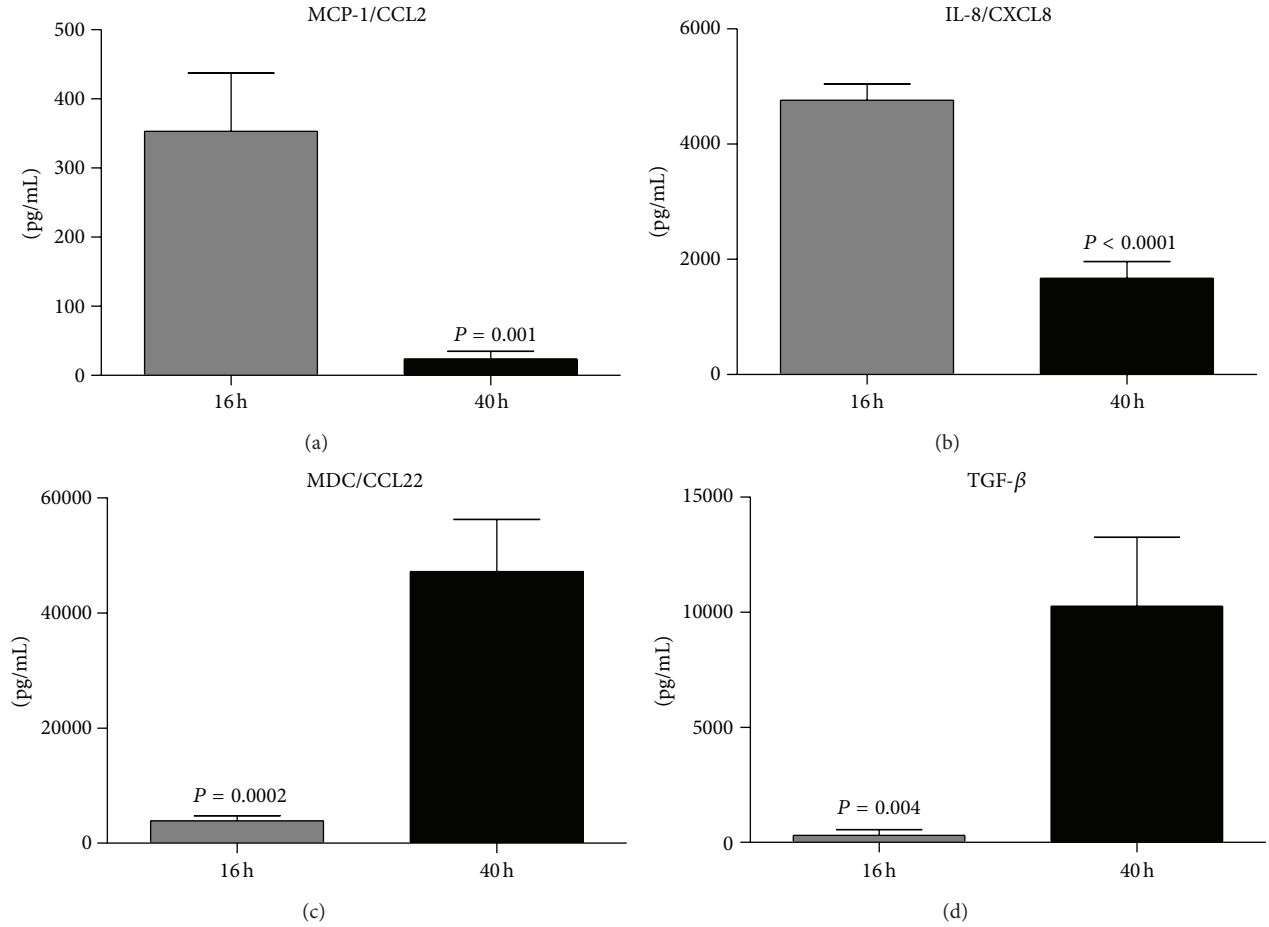

Figure 4: Enzyme-linked immunosorbent assay (ELISA) detection of inflammatory regulators in blister fluid: (a) MCP-1/CCL2, (b) IL-8/CXCL8, (c) MDC/CCL22, and (d) TGFβ. Blister fluid from $n = 10$ individuals was analyzed by ELISA in duplicate, at each of the timepoints. Results are expressed as mean ± S.E.M. P values represent statistical differences between 16-hour and 40-hour timepoints analyzed by unpaired t test.

process, as proposed previously [28, 29]. The sequence by which leukocytes were recruited into blisters was broadly similar to that previously described in an incisional wound model in humans, with neutrophils predominating at the early timepoint followed by a wave of monocyte/macrophage recruitment [13]. Neutrophil apoptosis and phagocytosis by macrophagic Reiter's cells were also captured in second day blisters. Whereas one-day-old cantharidin blisters have been previously used to demonstrate impaired neutrophil migration in Crohn's disease [30], extending blisters into a second day might enable impaired clearance of apoptotic cells to be studied in autoimmune diseases. Lymphocytes accumulated at 40 h, but it was not possible to examine later timepoints, since blisters became too fragile. It was notable that T cell chemoattractants implicated in wound repair [13], like MIG/CXCL9, and T cell cytokines, such as interferon-γ, were absent, but again it was not possible to examine any later timepoints in this study.

One of the most striking features of our data was the rapidity with which proinflammatory mediators were lost in the intervening 24 hours between the first and second sampling points. Bearing in mind that we studied an accumulation model, one would have expected cytokines present at 16 h to be still there at 40 h, unless they had been actively broken down, quenched, or extruded from the blister. We observed that concentrations of MCP-1/CCL2, IL-8/CXCL8, and TNFα each fell >1 log between 16 h and 40 h. The rapid loss of inflammatory mediators is consistent with the possibility of proteolytic degradation or quenching by proteoglycans, which has been described for chemokines [31–33], but either of these options remains to be proven. There was also a rapid switch to immunoregulatory factors in the blister model, with high levels of TGFβ detected at 40 h. This is quicker than has been reported previously using full thickness wound models where TGFβ peaks at 7 days [34], but the speedier transition to the resolution phase may have been be due to the fact that cantharidin skin blisters do not penetrate the dermis, obviating the need for a prolonged inflammatory or granulation step. The two-day blister model may be well suited for examining impaired wound healing in diabetes, where TNFα dysregulation has been shown to drive inflammatory and apoptotic processes, as well as impairing signalling in wounds [35, 36].

There was a marked increase in cells of the monocyte/macrophage lineage at the 40 h timepoint, and $CD163^+$

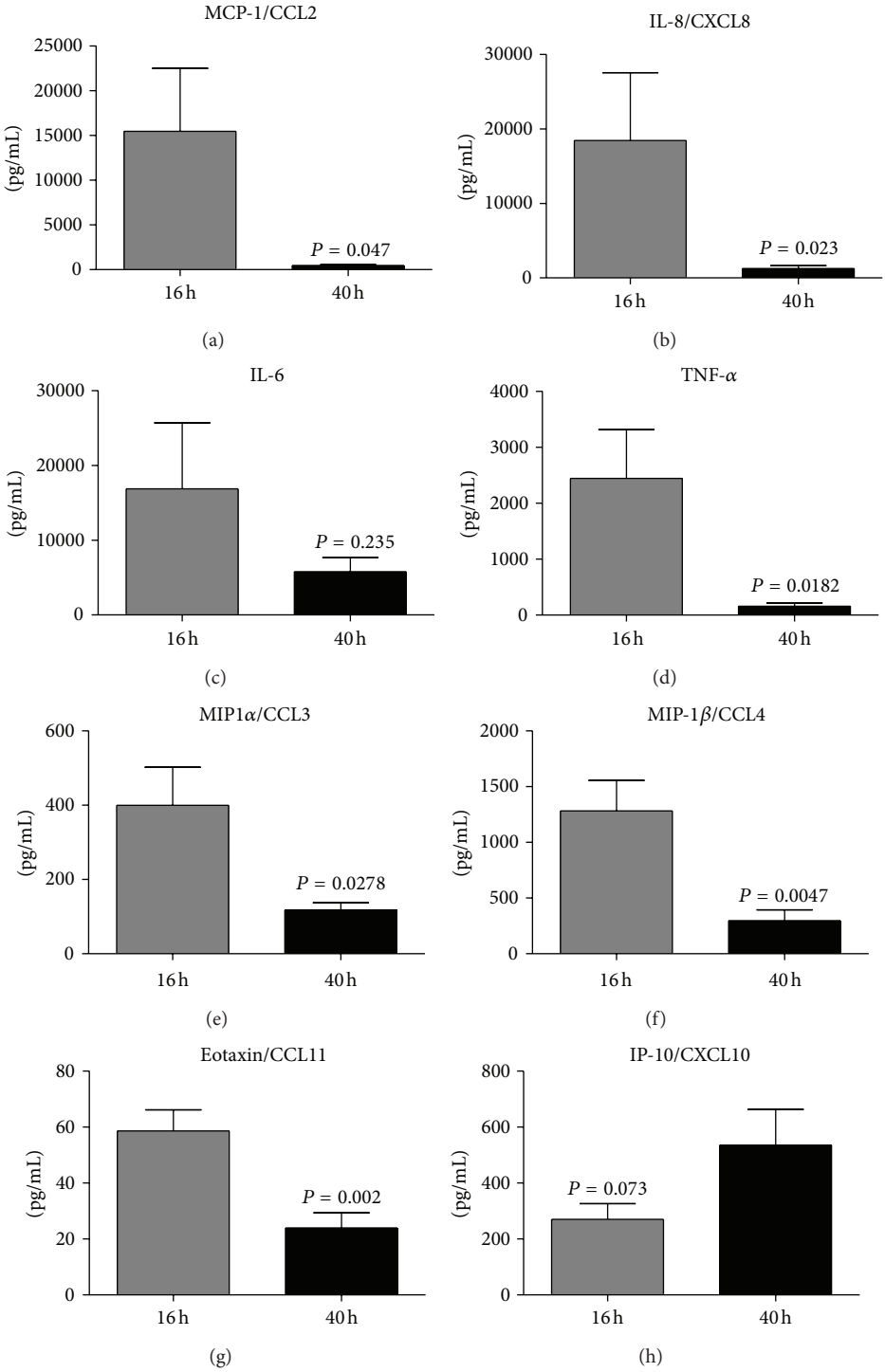

Figure 5: Multiplex bead array (Luminex) analysis of inflammatory regulators in blister fluid: (a) MCP-1/CCL2, (b) IL-8/CXCL8, (c) IL-6, (d) TNFα, (e) MIP-1α/CCL3, (f) MIP-1β/CCL4, (g) Eotaxin/CCL11, and (h) IP-10/CXCL10. Blister fluid from $n = 10$ individuals was analyzed by multiplex bead array at each of the timepoints. Results are expressed as mean ± S.D. P values represent statistical differences between 16-hour and 40-hour timepoints analyzed by unpaired t test.

macrophages accounted for almost 50% of this cell population. The conversion into the "resolving macrophage" phenotype was quicker than previously reported in a subcutaneous rat model, which required 21 days to achieve a similar 43%–56% conversion into the ED2 phenotype [37]. Again, the likely explanation lies with the fact that the skin blister model provides a less complicated lesion than the skin implant model studied in rats and the transition to the resolution phase is quicker. Previous work has revealed an important role for free haemoglobin driving CD163 expression via

an IL-10 feedback loop [10]. However, this mechanism does not appear to operate in skin blisters since there was no hemorrhagic component detected in skin blisters and IL-10 was undetectable at either timepoint [38]. The lack of IL-10, IL-4, and IL-13 detected in our model is consistent with the same observation made in animal wounds [39]. The identity of the polarising factor for CD163 conversion in skin is the aim of ongoing investigation, since CD163 expression is linked to beneficial antioxidant and anti-inflammatory pathways in macrophages [7, 10, 12, 40–43].

While cantharidin skin blisters present fewer ethical implications for wound research in humans than incisional or excisional models of wound healing, they also present some limitations. The rapidity of proinflammatory cytokine switching and shift to the $CD163^+$ phenotype is clearly at odds with past research in full thickness injury models which exhibited a slower timecourse. Hence, it is unclear whether the molecular details of wound repair learned in the skin blister model can be extended to deeper wounds. Nonetheless, neutrophil apoptosis and phagocytic clearance by macrophagic cells with accompanying TGFβ release were reproduced in the skin blister model, suggesting that the major hallmarks of inflammatory resolution were present.

In conclusion, we have demonstrated that skin blisters extended into a second day reproducing the major features of the resolving phase in wound healing. The usefulness and safety of using cantharidin skin blisters have already been demonstrated for discriminating immediate and delayed inflammatory responders and in studies of the systemic inflammatory response to cardiopulmonary bypass and patients with inflammatory diseases, such as Crohn's [27, 30, 44]. Extension of the cantharidin skin test into the second day may find therapeutic application in evaluating wound healing treatments or proresolving anti-inflammatory interventions [45].

Conflict of Interests

The authors declare no conflict of interests.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2013, Article ID 980327, 7 pages
http://dx.doi.org/10.1155/2013/980327

Research Article

Haptoglobin Genotype-Dependent Anti-Inflammatory Signaling in CD163$^+$ Macrophages

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Received 15 March 2013; Accepted 3 April 2013

Academic Editor: David A. Hart

Intraplaque hemorrhage causes adaptive remodelling of macrophages towards a protective phenotype specialized towards handling iron and lipid overload, denoted Mhem. The Mhem phenotype expresses elevated levels of hemoglobin (Hb) scavenger receptor, CD163, capable of endocytosing pro-oxidant free Hb complexed to acute phase protein haptoglobin (Hp). It is notable that individuals homozygous for the Hp 2 allele (a poorer antioxidant) are at increased risk of cardiovascular disease compared to the Hp 1 allele. In this study, we examined whether scavenging of polymorphic Hp:Hb complexes differentially generated downstream anti-inflammatory signals in cultured human macrophages culminating in interleukin (IL)-10 secretion. We describe an anti-inflammatory signalling pathway involving phosphatidylinositol-3-kinase activation upstream of Akt phosphorylation (pSer473Akt) and IL-10 secretion. The pathway is mediated specifically through CD163 and is blocked by anti-CD163 antibody or phagocytosis inhibitor. However, levels of pSer473Akt and IL-10 were significantly diminished when scavenging polymorphic Hp2-2:Hb complexes compared to Hp1-1:Hb complexes ($P < 0.05$). Impaired anti-inflammatory macrophage signaling through a CD163/pAkt/IL-10 axis may thus represent a possible Hp2-2 disease mechanism in atherosclerosis.

1. Introduction

Intraplaque hemorrhage is a common complication of atherosclerosis and is linked to plaque progression, especially in diabetes [1–4]. However, work from independent groups has demonstrated that macrophages at zones of hemorrhage may exert some level of homeostatic control through adaptive remodelling towards an Mhem phenotype capable of handling iron and lipid overload [5–7]. Scavenging of haptoglobin:hemoglobin (Hp:Hb) complexes via CD163 is part of this adaptive process, linked to secretion of anti-inflammatory cytokine interleukin (IL)-10 and elevation of heme oxygenase (HO)-1 [8–10]. Analogous protective pathways are evoked by free diffusion of purified heme or by phagocytosis of damaged erythrocytes via CD204, leading to the proposal that a final common pathway is instigated by accumulation of intracellular heme capable of transcriptionally activating genes involved in iron handling and cholesterol efflux (e.g., HO-1 and liver X receptor) via transcription factors Nrf2 and activating transcription factor (ATF)-1 [11, 12]. Although the atheroprotective properties of the Mhem macrophage phenotype is therefore well established, the role of proximal signalling pathways linked to anti-inflammatory IL-10 secretion via CD163-dependent uptake of Hb:Hp remains to be fully understood.

In addition to IL-10, immunoregulatory IL-6 cytokine has been consistently reported downstream of CD163 [8, 13, 14]. However, the earliest IL-6 studies using cross-linking anti-CD163 antibodies may not have adequately discriminated between surface versus endocytosis-dependent effector pathways [13, 14]. Whether downstream signals require simple cross-linking of CD163 at the cell surface or phagocytosis

of the entire Hp:Hb complex is an important distinction, since Hp2-2:Hb binds more avidly than Hp1-1:Hb to CD163 at the surface but, conversely, is more poorly internalised into the cell [15, 16]. More recent investigations employing native Hp:Hb ligand appeared to suggest poor or even lack of dependence on CD163 for IL-6 or IL-10 signalling pathways, depending on the type of polymorphic haptoglobin variant employed [17, 18]. Since the haptoglobin 2 allele is linked to a host of adverse clinical cardiovascular events, [19–23] it is important to understand Hp genotype-dependent disease mechanisms in $CD163^+$ macrophages in greater detail, to guide informed interdictions in vulnerable individuals.

Here we have examined IL-10 signalling pathways during scavenging of polymorphic Hp2-2:Hb versus Hp1-1:Hb complexes in $CD163^+$ human monocyte-derived macrophages. We identify a specific Akt/IL-10 pathway that is comparatively underinduced during the scavenging of Hp2 complexes.

2. Materials and Methods

2.1. Reagents and Antibodies. Human Hb (A_o), human Hp (phenotypes 1-1 and 2-2), and colchicine were purchased from Sigma-Aldrich (Poole, UK). Anti-human CD163 monoclonal antibody clones RM3/1, Ki-m8, and 5C6-FAT were purchased from Bachem (Merseyside, UK), clone GHI/61 from BD Pharmingen (Oxford, UK), and clone Ber-MAC3 from Dako (Cambridge, UK). Polyclonal anti-Akt and anti-phosphoAkt (Ser473) antibodies were purchased from Cell Signalling Technology, Inc. (Beverley, MA). The phosphoinositide-3-kinase (PI-3K) inhibitor, Ly294002, was purchased from Alexis Corporation (Bingham, UK). Endotoxin determinations were made using the GCL-1000 LAL chromogenic endpoint assay (Cambrex Bio Science, Wokingham, UK).

2.2. Hb:Hp Treatment of Macrophage Cultures. Human monocytes were isolated from venous blood and differentiated into $CD163^+$ macrophages *in vitro* as described [8]. Hb:Hp complexes were generated by dissolving equimolar amounts of Hb and Hp in growth medium. Hb, Hp, or Hb:Hp were added at final concentrations of 1 mg/mL unless otherwise stated to monocyte/macrophage cultures prior to incubation for 24 hours and collection of supernatants and/or cell lysates for IL-10 and Akt analysis, respectively. Hb or Hp batches containing detectable endotoxin (>5 pg/mL) were discarded. Supernatants and cell lysates were stored in aliquots at −70°C prior to analysis. In some experiments, the PI-3K inhibitor Ly294002 was added at 50, 25, 12.6, and 6.25 μmol/L final concentrations. Actinomycin D and cycloheximide were added at a concentration of 1 μg/mL and colchicine at a concentration range between 10 and 1.25 μmol/L. None of the inhibitors at the concentrations used exhibited significant macrophage cellular cytotoxicity [24]. Anti-CD163 monoclonal antibodies were added at a final concentration of 20 μg/mL, previously shown to be neutralizing [8].

2.3. Enzyme Linked Immunosorbent Assays. IL-10 concentrations in culture supernatants were determined by ELISA technique (Quantikine; R&D Systems, Abingdon, UK) according to the manufacturer's recommendations. Phospho-Akt levels in cell lysates, collected in buffer consisting of 1% Triton X-100, 25 mmol/L sodium deoxycholate, 150 mmol/L NaCl, 50 mmol/L Tris pH 7.4, 4 mmol/L EDTA, 200 μmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, and 5% protease inhibitor cocktail (Sigma Aldrich), were measured by pSer473 Akt kit (Biosource, Camarillo, CA) according to manufacturer's instructions. All samples were measured in duplicate and results expressed as mean cytokine concentration (pg/mL) or pAkt activity (U/mg) ± SEM from $n = 3$ experiments.

2.4. Western Blot Analysis. Monocytes/macrophages were lysed in buffer, as described for the phospho-Akt ELISA, and proteins were separated by SDS-PAGE on a 12.5% gel prior to transfer to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Equal loading of lanes was confirmed by estimation of lysate protein content using the Bio-Rad D_c protein assay (Bio-Rad, Hercules, CA). Induction of pSer473Akt was established relative to total Akt by probing blots with polyclonal antibody against pSer473Akt, followed by stripping and reprobing with total Akt. Blots were developed with an enhanced chemiluminescence substrate (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK).

2.5. Statistical Analysis. Statistical comparisons between Hp1-1:Hb and Hp2-2:Hb groups were performed using an unpaired Student's t-test. Multiple group comparisons of pSer473Akt activity were performed using a one way ANOVA with a Student-Newman-Keuls posttest. Statistical analysis was performed using GraphPad Prism (GraphPad Prism Software, Inc., San Diego, CA), and significance was assumed at $P < 0.05$.

3. Results

3.1. Impaired IL-10 Secretion following CD163-Dependent Scavenging of Hp2-2:Hb. IL-10 induction has been linked to CD163 receptor engagement [8, 13, 14, 17, 18]. Differential IL-10 responses by $CD163^+$ macrophages were therefore examined in the presence of polymorphic Hp:Hb complexes. The addition of Hp:Hb complexes across a range of concentrations to *in vitro* differentiated $CD163^+$ macrophages revealed significantly impaired IL-10 induction in the case of Hp2-2. At concentrations within the range of plasma haptoglobin (0.1–2 g/L) [18, 25], the IL-10 response to Hp2-2:Hb was significantly diminished compared to Hp1-1:Hb (e.g., at 1 g/L: 3509 ± 169 pg/mL versus 6739 ± 678, $P < 0.01$; Figure 1(a)). The timecourse of IL-10 induction to either type of Hp:Hb complex was relatively slow, with a peak at 48 h (Figure 1(b)). Consistent with the slow kinetic, IL-10 secretion required prior transcription and protein synthesis, as it was abrogated in the presence of actinomycin D or cycloheximide (data not shown). In agreement with previous observations [8, 15, 17] and again repeated here, IL-10 induction required formation of a Hp:Hb protein complex, since neither Hb alone, nor Hp alone yielded significant IL-10 secretion (Figure 1(c)). A panel

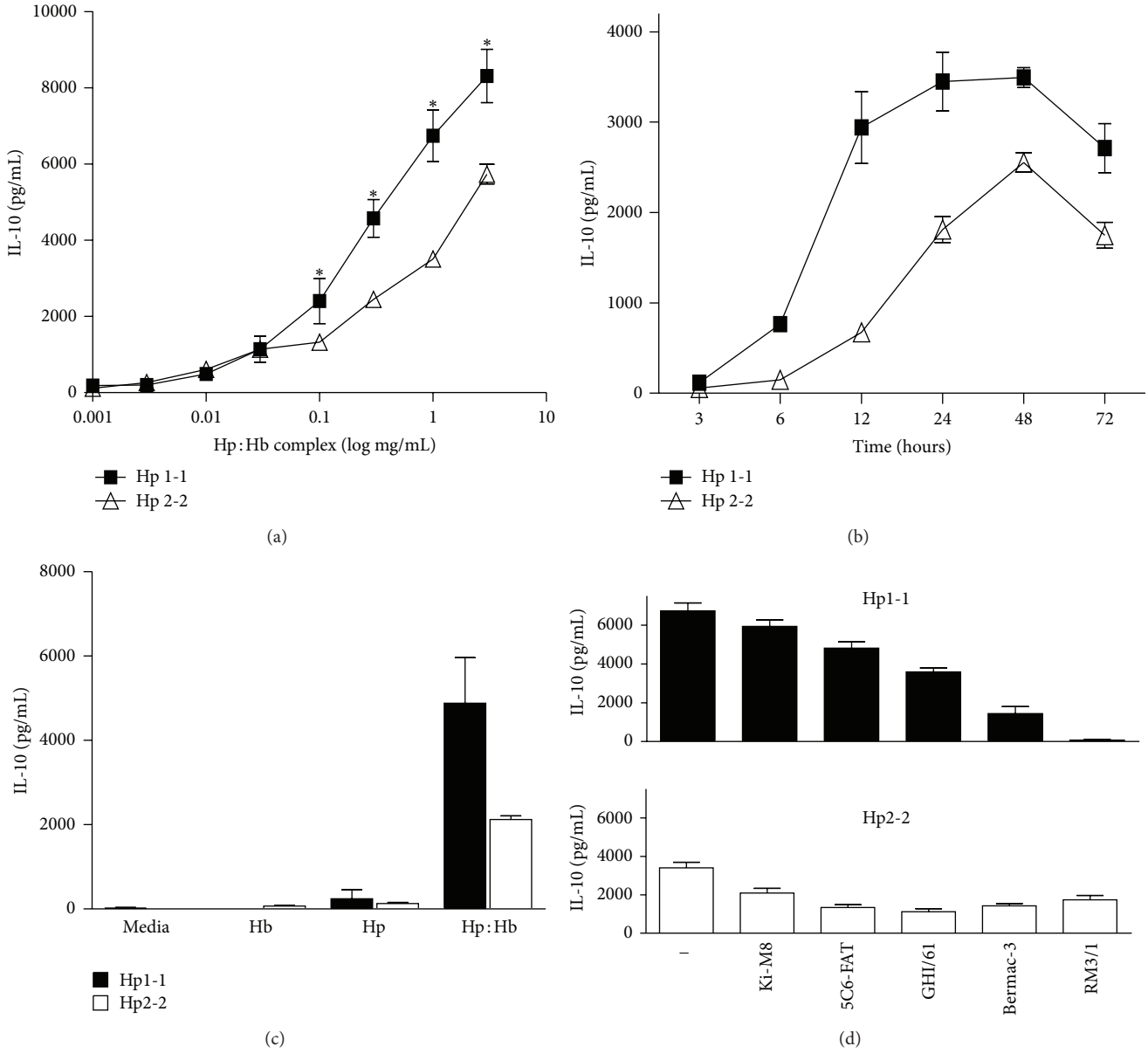

Figure 1: CD163-dependent IL-10 secretion following scavenging of polymorphic Hp:Hb complexes. (a) Effect of increasing concentrations of polymorphic Hp:Hb complexes on IL-10 secretion by $CD163^+$ human monocyte-derived macrophages at 24 h. (b) Timecourse of IL-10 secretion following addition of polymorphic Hp:Hb complexes at 1 mg/mL. (c) IL-10 secretion by $CD163^+$ macrophages stimulated with Hb alone, Hp1-1, Hp2-2, or polymorphic Hp:Hb complexes (1 mg/mL) at 24 h. (d) Effect of a panel of anti-CD163 antibodies (20 μg/mL) on IL-10 secretion induced by polymorphic Hb:Hp complexes (1 mg/mL) at 24 h. All IL-10 assays were carried out in duplicate, and results are expressed as mean IL-10 (pg/mL) ± SEM from $n = 3$–9 experiments. $^*P < 0.01$.

of five anti-CD163 antibodies showed a similar inhibitory profile to either type of complex with the notable exception of the function blocking anti-CD163 antibody RM3/1 [8]. RM3/1 abolished IL-10 secretion in response to Hp1-1:Hb but only weakly inhibited the response to Hp2-2:Hb (Figure 1(d)).

3.2. Signaling Pathways Linked to CD163-Dependent Hp:Hb Scavenging. The IL-10 response to either type of Hp required phagocytosis of complexes, since it was blocked in the presence of the phagocytosis inhibitor colchicine (Figure 2(a)). A screen of PI-3K, PKC, p42/44 MAP kinase, and p38 MAP kinase signaling pathway inhibitors revealed that the IL-10 response to Hp:Hb complex scavenging was dose dependently inhibited by the PI-3K inhibitor LY294002 (Figure 2(b)). This was true for Hp2-2:Hb as well as Hp1-1:Hb complexes. The pan-PKC inhibitor bisindolylmaleimide, the p42/44 MAP kinase inhibitor U0126, and p38 MAP kinase

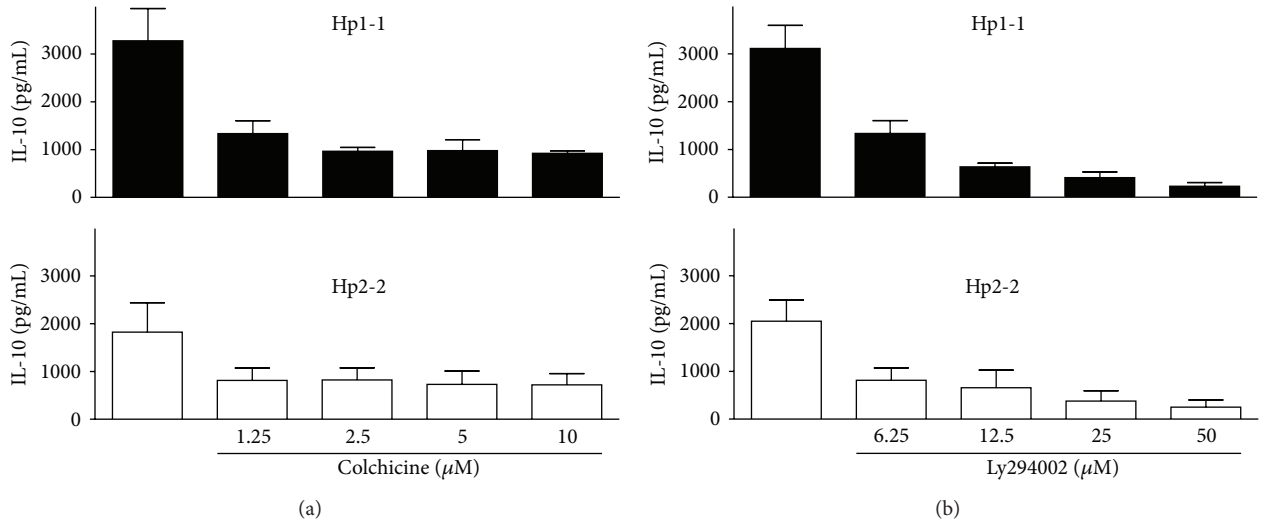

Figure 2: Effect of pharmacological inhibitors on Hp dependent IL-10 secretion. Effect of (a) colchicine (1.25–10 μmol/L) and (b) Ly294002 (6.25–50 μmol/L) on IL-10 release by CD163$^+$ human monocyte-derived macrophages stimulated with polymorphic Hp:Hb complexes (1 mg/mL) for 24 h. Results are expressed as mean IL-10 (pg/mL) ± SEM from n = 3–4 experiments.

inhibitor SB203580, used at a concentration range known to inhibit cytokine secretion in the same cell preparation against calcific microcrystals [24], had no effect on IL-10 secretion (data not shown).

To further explore signaling pathways linked to CD163 downstream of PI-3 kinase, phosphorylation of Akt was investigated. Phosphorylation of Akt at serine 473 (pSer473Akt) was examined by Western blot as well as phosphoSer473Akt-specific ELISA: both assays showed phosphorylation of Ser473Akt and this was inhibited significantly by anti-CD163 antibody RM3/1 or colchicine (Figures 3(a) and 3(b)). Akt phosphorylation required upstream PI-3Kinase activation, as it was abrogated in the presence of PI-3Kinase inhibitor Ly294002 (Figures 3(a) and 3(b)). Akt phosphorylation was markedly impaired following scavenging of Hp2-2:Hb complexes compared to Hp1-1:Hb, across a broad concentration range (e.g., at 1 g/L: 0.20 ± 0.02 U/mg versus 0.50 ± 0.10, $P < 0.05$; Figure 3(c)).

4. Discussion

The central role of Hp in complexing free Hb and mediating its clearance through CD163 led us to consider whether the Hp2-2 genotype, clinically associated with cardiovascular complications, may show impaired anti-inflammatory signaling engagement downstream of CD163. The model chosen for this work (human monocytes differentiated for 7 days in culture) benefits from the absence of exogenously added differentiating agents (e.g., steroids) and has been validated against human macrophages differentiated *in vivo*: equivalent functional responses to Hp:Hb scavenging were noted in CD163$^+$ macrophages recovered from human skin blisters during the cutaneous inflammatory response to cantharidin [8]. The CD163/Akt/IL-10 axis identified here adds to a growing understanding that homeostatic signalling pathways may be triggered in CD163$^+$ macrophages during hemoglobin scavenging and that these are impaired in the case of polymorphic Hp2-2 protein. Our data add a cellular dimension to the innate antioxidant properties recognised for Hp variants (Hp1-1 superior to Hp2-1 superior to Hp2-2) [4, 26, 27] and suggest a plausible new disease mechanism linking Hp2-2 with atherothrombosis.

Our findings are consistent with previous reports on the relative inability of Hp2-2:Hb complexes to trigger IL-10 responses [17, 18] and may explain why previous reports noted an apparent lack of CD163 receptor usage by polymorphic Hp2-2:Hb variants [17, 18]. In those studies, RM3/1 was employed as the blocking antibody. Here we confirm that RM3/1 is the most potent blocking antibody against Hp1-1:Hb induced IL-10 secretion, out of a screen of five anti-CD163 antibodies (RM3/1, Ki-M8, 5C6-FAT, GHI/61, and Bermac-3). However, RM3/1 exhibited only weak blocking against Hp2-2:Hb complexes. Hence, it is not likely that Hp2-2:Hb signals through a different (unknown) receptor pathway. The evidence presented here with other blocking antibodies (Bermac3 and GHI/61) as well as the phagocytosis inhibitor colchicine strongly suggests that both types of Hp:Hb complexes are endocytosed via CD163.

Results from the panel of blocking antibodies also have implications for the way in which signals are generated through CD163. The elegant structure-function analysis of CD163 protein [28], which combined ligand binding studies in solution with a comprehensive epitope map of ten anti-CD163 antibodies, failed to detect any direct ligand-blocking property for RM3/1. Only Ki-m8 and Edhu-1, mapping to the ligand binding scavenger receptor cysteine-rich (SRCR) domain 3, directly inhibited Hp:Hb binding to CD163 in solution. Since these same two antibodies behaved as agonistic antibodies when added to cells [8, 13, 14], this supports the concept that cross-linking of two or more ligand-binding

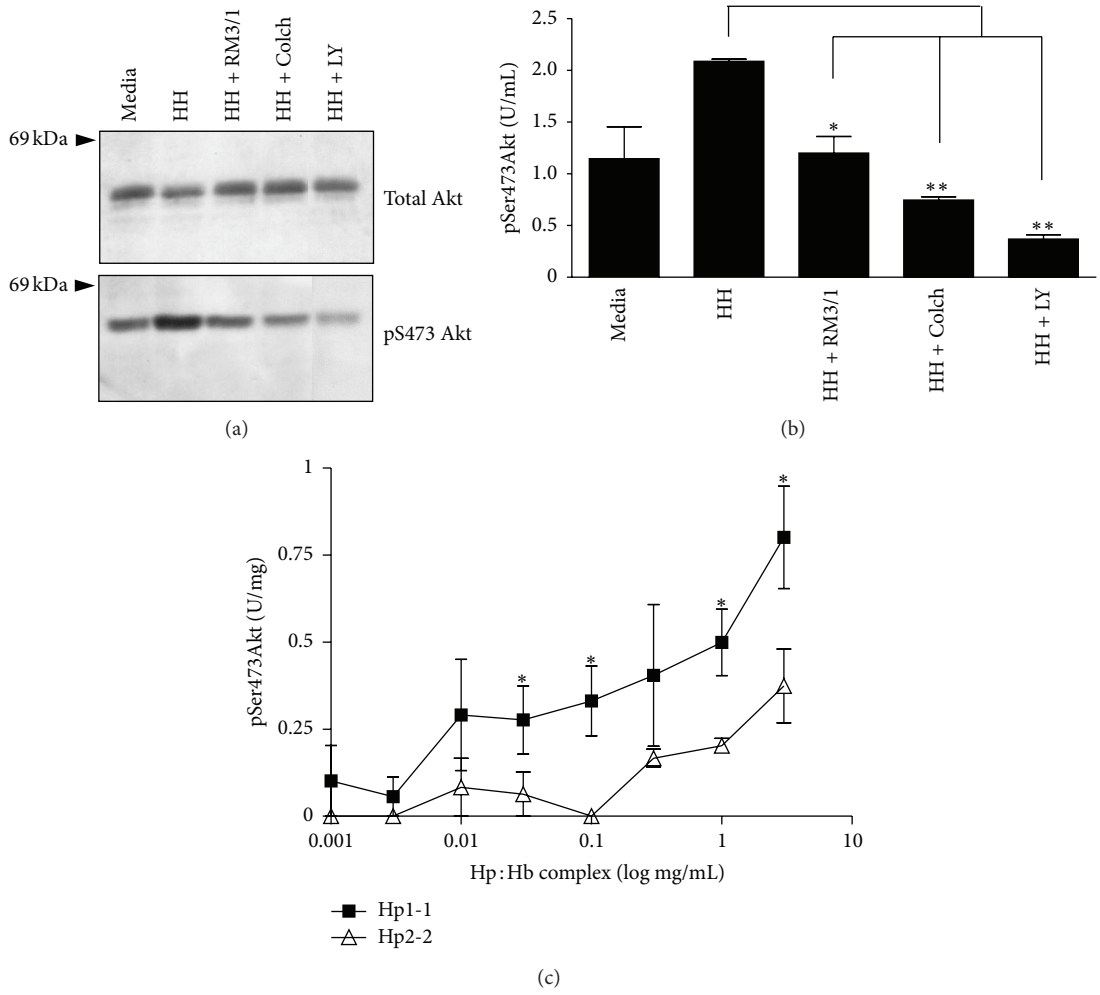

Figure 3: Hp:Hb scavenging-dependent Akt phosphorylation. (a) Representative Western blot showing phosphorylated Akt (pSer473) and total Akt in lysates from macrophages stimulated with Hp1-1:Hb (1 mg/mL; HH in legend) for 20 minutes in the presence and absence of blocking anti-CD163 antibody clone RM3/1 (20 μg/mL; RM3/1 in legend), colchicine (2.5 μmol/L; colch in legend), or phosphoinositol 3-kinase inhibitor Ly294002 (12.5 μmol/L; Ly in legend). (b) Quantitative ELISA determination of Akt (pSer473) phosphorylation in cell lysates from macrophages treated as in (a) above. (c) Comparison of Hp2-2:Hb versus Hp1-1:Hb scavenging on Akt phosphorylation in cell lysates, as measured by pSer473Akt-specific ELISA. Results in (b) and (c) are expressed as pSer473Akt (U/mg) ± SEM from three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

domains on CD163 must be required for signal generation, as originally proposed [15]. Out of the panel of ten anti-CD163 antibodies previously mapped, RM3/1 uniquely mapped to the last SRCR domain (domain 9, immediately proximal to the plasma membrane) [28]. This suggests that its function blocking property relates to an ability to block dimerization of CD163 at the membrane following cross-linking with native Hp1-1:Hb holo-dimer. Since Hp2-2 exists as a larger multimer, consisting of 3–8 subunits, this might explain why RM3/1 only partially blocks IL-10 signal production in response to the multimeric complex.

We describe a hitherto unappreciated signalling pathway following Hp:Hb complex endocytosis, requiring Ser473Akt phosphorylation and culminating in IL-10 secretion. We recognise the limitations posed through the use of a pharmacologic inhibitor of PI-3 kinase, but phosphorylation of Ser473Akt was verified both at Western blot and quantitative intracellular ELLISA levels. Adenoviral infection approaches to further dissect the signalling pathway were not successful, since infection even with empty vectors triggered phosphorylation of Akt, therefore ruling out this approach (data not shown). Past studies that used adenovirus to investigate signalling pathways in macrophages used differentiating agents not compatible with the $CD163^{+}$ endpoint and our cells may therefore have behaved differently [29].

Studies carried out prior to the identification of Hp:Hb as ligand for CD163 employed a cross-linking anti-CD163 antibody, EDhu-1, and reported IL-6 secretion that was casein kinase II and PKC dependent [13, 14]. Secretion of IL-6 in our hands, in response to Hp:Hb, was not specific to CD163, since it did not require Hp complex formation with Hb, did not require an intact microtubular assembly for phagocytosis, and was not blocked by function-blocking antibodies or a PI-3K inhibitor (data not shown). Our own

results do not therefore support IL-6 as an effector molecule downstream of CD163, whereas IL-10 was confirmed and was Hp genotype dependent. Alternative receptor mechanisms and endotoxin contamination were excluded as confounding sources of intracellular signals. Mac-1 was eliminated as a possible alternative candidate receptor for haptoglobin [30]. Endotoxin levels in all cell cultures were below 5 pg/mL and inhibition of IL-10 secretion by phagocytic inhibitors also ruled out endotoxin as a possible trigger. Our previous work has shown that above a threshold concentration of >10 ng/mL of Hp:Hb complexes, all or none commitment to the $CD163^+$ phenotype occurs via an IL-10/CD163 feedback loop [31]. This scenario is consistent with observations that $CD163^+$ macrophages closely associate with areas of hemorrhage [10, 31].

In summary, the present study has identified an PI-3/Akt signaling pathway contributing to IL-10 secretion via $CD163^+$-dependent hemoglobin scavenging, a pathway that is impaired during the scavenging of polymorphic Hp2-2 complexes. This adds not only to our understanding of cardiovascular disease susceptibility for Hp2-2 but may also be relevant to the protection of the vasculature and kidneys from heme-mediated oxidative injury secondary to hemolysis [32–35], especially in predisposing conditions like diabetes, sickle cell anemia, or leukaemia treatment [16, 36, 37]. In conjunction with previous observations in atherosclerotic plaques [5, 12, 31], we propose that $CD163^+$ macrophages may act as a homeostatic brake on plaque inflammation secondary to hemorrhage, in an Hp genotype dependent manner.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by the British Heart Foundation and the Barbados Diabetes Foundation.

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Hindawi Publishing Corporation
International Journal of Inflammation
Volume 2012, Article ID 697592, 9 pages
doi:10.1155/2012/697592

Review Article

The Immune Response: Targets for the Treatment of Severe Sepsis

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Received 3 September 2012; Accepted 6 November 2012

Academic Editor: Paulo Roberto B. Evora

The clinical process of severe sepsis is characterized by extreme inflammation interlinked with potent stimulation of the coagulation cascade often followed by a state of relative immune paralysis. In this paper, we will review many of the potential therapies directed at various steps along the inflammatory cascade from modulation of inflammatory mediators eliciting the immune response, alteration of the host's immune response in both a stimulatory and depressive manner, and taming the overexuberant coagulation response triggered by the fierce coagulation-inflammation cycle. Finally, we will discuss further opportunities for research to improve our ability to design effective therapies.

1. Introduction

The syndrome of severe sepsis is described as a hyper-immune response to one of many infectious insults. It results in an overwhelming surge of cytokines leading to the clinical syndrome of hypotension, multiple organ failure and, sometimes, death [1]. This uncontrolled, hyperimmune response is often accompanied by a state of relative immune paralysis caused by apoptosis of immune cells and high levels of anti-inflammatory cytokines which function to inhibit lymphocytes and macrophages and suppress the production of proinflammatory cytokines. This immune paralysis is postulated to cause the delayed mortality seen in some septic patients due to their inability to oppose and eliminate infections. The balance between hyperimmune response and immune paralysis varies based on patient as well as throughout the course of illness within the same patient [1–3].

Sepsis continues to be a significant cause of illness and death worldwide. In the United States alone, it is estimated that it affects more than 750,000 people annually and causes more than 210,000 deaths. Approximately 40% of all intensive care unit patients become septic at some time during the ICU course [3].

To date, the sole universally agreed upon treatment for sepsis includes fluids, vasopressors, and source control as defined by the International Surviving Sepsis Campaign Guidelines Committee in 2008. While the therapeutic monitoring goals remain controversial, this strategy of fluid administration and, if needed, vasopressor infusion to restore organ perfusion, source control with a focus on early administration of appropriate broad-spectrum antibiotics, and maximizing oxygen delivery with supplemental oxygen and red blood cell transfusion as indicated is thought to be the most effective approach [4, 5]. Outside of these measures, numerous supplementary strategies have been evaluated without discovery of the perfect antidote.

2. Inflammatory Mediators

Decades ago, unfruitful attempts were made to create antibodies with the potential to bind and to prevent inflammatory bacterial components from triggering the

hyperinflammatory response of sepsis. Lipopolysaccharide (LPS), a primary mediator in gram-negative sepsis, was the target of researchers as early as the 1980s. Clinicians tested E5 and HA1A, both anti-LPS monoclonal antibodies, as treatments for septic patients. In initial studies, both antibodies showed encouraging results in small subsets of patients. Fink showed improvement in mortality in patients with culture-proven gram-negative bacteremia when treated with HA1A [6]. Ziegler et al. showed improved mortality with the use of HA-1A therapy in 200 patients with proven gram-negative sepsis. The 343 septic patients without culture proven gram-negative bacteremia showed no treatment benefit [7]. Greenman et al. evaluated E5 in 1991 and showed improved mortality and resolution of organ failure in a subgroup of patients not in shock at the time of study entry [8]. In a follow-up study, Bone et al. evaluated 530 patients with suspected or proven gram-negative sepsis and did not find a difference in mortality but demonstrated improvement of organ failure resolution in those treated with E5 as well as prevention of adult respiratory distress syndrome and central nervous system organ failure [9]. Unfortunately, further studies of these therapies in larger clinical trials including more than 1,000 patients each were unable to confirm efficacy [10–12].

More recently, this approach has been revisited with the concept of inhibiting toll-like receptor 4 (TLR-4) which is expressed on the surface of immune cells and binds LPS and other ligands to initiate an intracellular signaling cascade resulting in the release of proinflammatory cytokines [13]. The therapy, TAK-242, functions as a signal inhibitor of the TLR-4 pathway acting after TLR-4 binds with LPS. In septic animal models an improved survival associated with decreased levels of inflammatory cytokines has been shown with the use of this therapy. Furthermore, its use in healthy volunteers prior to instillation of LPS also resulted in decreased levels of inflammatory cytokines when these patients were given an LPS challenge. In 2010, Rice et al. evaluated TAK-242 in a randomized, placebo-controlled trial of patients with severe sepsis and shock or respiratory failure. High-dose and low-dose treatment regiments were compared to placebo with primary endpoints of change in IL-6 level and 28-day mortality rate. This trial was terminated after enrollment of 274 patients failed to show suppression of IL-6 levels. Evaluation of the treated patients showed no difference in 28-day mortality compared to placebo, however, there was a trend toward improved survival in those with both shock and respiratory failure who were in the higher treatment dose cohort [14]. It may be that this therapy could be effective in patients with a higher severity of illness, as suggested by the trend towards improved survival of the patients with both respiratory failure and shock. Furthermore, the mean time from onset of shock or respiratory failure to initiation of TAK-242 therapy was 19 hours. The dynamic nature of the immune response has been well described and it could be postulated that the delay of 19 hours is too long for the treatment to have the ability to suppress the immune response.

3. Steroids

Steroids act early in the inflammatory cascade eliciting a wide range of effects via broad suppression of the immune system and are hypothesized to provide benefit as a supplementary treatment of sepsis. Steroids function by inhibiting production of proinflammatory cytokines (TNF-α, IL-1, IL-2, IL-6, and IFN-gamma), chemokines, bradykinins, and eicosanoids. Simultaneously, they increase anti-inflammatory mediators (IL-10, IL-1 receptor antagonists, and TNF receptor antagonists), inhibit inducible nitric oxide synthase, decrease migration of inflammatory cells to sites of inflammation, and reduce the function of inflammatory cells [1]. It is further postulated that steroids increase the expression of adrenergic receptors in the vasculature. These receptors are downregulated in septic shock and, theoretically, increasing their expression allows the vasculature to respond to the high levels of circulating cortisol [2].

Initially, studies were done using high-dose steroids (e.g., 30 mg/kg methylprednisolone) with the goal of broad suppression of the body's overreactive inflammatory response [15–17]. These studies failed to show benefit and even showed a trend towards harm including increased mortality due to secondary infections in those treated with steroids, thus, causing steroid therapy use to decrease in the early 1990s [2, 17]. The use of steroids was revived in the mid 1990s with the target of treating relative adrenal insufficiency with the use of replacement, low-dose glucocorticoids. The treatment with low-dose steroids is thought to improve vascular response to endogenous and exogenous catecholamines via the upregulation of adrenergic receptors in the vasculature. While avoiding the substantial immune system blockade, this lower dose is thought to maintain some anti-inflammatory effects via preventing release of proinflammatory cytokines and activation of endothelial cells and neutrophils to decrease sepsis triggered clotting disorders [15, 16].

Small studies done in the late 1990s showed trends toward improvement in hypotension and mortality with the low-dose steroid treatment strategy. However, these studies were underpowered to detect clinically significant effects [1]. More recently, two large randomized, controlled trials have been published that further evaluated the effectiveness of steroid therapy. In 2002, a study completed by Annane et al. evaluated 300 patients with septic shock and showed improvement of refractory hypotension and a decrease in absolute mortality in patients with relative adrenal insufficiency treated with 7 days of hydrocortisone and fludrocortisone. This study also showed that adrenal-sufficient patients as defined as displaying a response to ACTH gained no benefit and trended towards harm from glucocorticoids [18]. Subsequently, the CORTICUS study published in 2008 by Sprung et al. compared the treatment of 499 septic patients with 11 days of hydrocortisone versus placebo. Contrary to the Annane study, the CORTICUS trial failed to show an improvement in mortality or reversal of shock in treated patients, regardless of ACTH response. They did, however, show a faster resolution of shock in those

patients who had shock resolution and were treated with hydrocortisone. Interestingly, they did show an increased incidence of superinfection in those treated with steroids [19]. The differences in the outcomes of these trials may be linked to several key differences between them. The populations studied included different timing of patient enrollment. The Annane study took patients up to 8 hours after onset of shock while the CORTICUS study extended their enrollment up to 72 hours after the onset of shock. Furthermore, the CORTICUS trial included all patients in shock while the Annane study restricted their study to only those who were both fluid and vasopressor refractory. Similarly, the patients in the Annane study were significantly more ill at baseline with a higher SAPS score and a higher mortality rate in the placebo groups than the CORTICUS trial (65% Annane trial versus 32% CORTICUS trial). It could be postulated that their conflicting results are a product of their differing patient populations as the Annane study evaluated a group of patients with a higher degree of illness and more refractory shock [4, 16].

Furthermore, the Annane and CORTICUS trials differed regarding the utility of the ACTH stimulation test. The Annane study showed that ACTH nonresponders were more likely to benefit from steroid therapy while the CORTICUS trial failed to replicate this finding [18, 19]. Given the challenges of measuring cortisol levels and the finding that the Annane trial showed an overall trend toward benefit of steroid therapy, regardless of ACTH responsiveness, the 2008 Surviving Sepsis Guidelines recommended that the ACTH stimulation test not to be used as a tool to guide the use of steroid therapy [4]. Known side effects of steroids including hyperglycemia, gastrointestinal bleeding, myopathy, and secondary infection have tempered the enthusiasm for steroid use. The CORTICUS trial, showing no efficacy of steroids reinforced these reservations when it also demonstrated an increase in episodes of superinfection with new sepsis and septic shock in those treated with steroids [16, 19].

Currently, the adult literature has not developed a standard of care in regards to steroid therapy. The Surviving Sepsis Guidelines recommend the use of steroids only in fluid and vasopressor refractory shock and do not recommend the use of the ACTH stimulation test based on low-grade and moderate-grade evidence, respectively [4]. Furthermore, they advise tapering the steroids when the state of shock resolves. Less data exists in regards to the pediatric population and the Surviving Sepsis Guidelines base recommendations on a retrospective review done by Markovitz et al. in 2005 [20]. This review showed that corticosteroid use in children with severe sepsis was an independent predictor of mortality. However, the nature of the study design does not allow for causal inference and the Surviving Sepsis Guidelines cite a weak recommendation based on low-grade evidence for the use of hydrocortisone only in children with catecholamine resistant shock and suspected or proven adrenal insufficiency [4]. Ideally, what is needed is a better means of determining the population of septic patients which have the best chance to benefit from steroid treatment while having the least risk of harm due to the side effects of the therapy.

4. Antagonism of Proinflammatory Cytokines

Knowledge of the inflammatory cascade and, more specifically, proinflammatory cytokines has allowed specific targets for immunosuppression including TNF-α and IL-1. TNF-α injection into animals has been shown to trigger a sepsis-like syndrome including hypotension, activation of the clotting cascade, significant organ dysfunction, and even death. Furthermore, increasing and persistently elevated levels of TNF-α are associated with nonsurvival in humans [21, 22]. Downstream effects of TNF-α include augmentation of the inflammatory cascade via elevation of multiple cytokine levels and upregulation of adhesion molecules on leukocytes, platelets, and endothelial cells. TNF-α also stimulates the coagulation system via activation of thrombotic and fibrinolytic pathways. Despite the deleterious effects of this overstimulation, it is evident that TNF-α plays a crucial role in the immune system because blockage of its activity in animal models has led to a worsened ability of the animal's immune system to clear microbes [21]. Due to its pivotal position in the inflammatory and coagulation systems that are known to cause the demise in sepsis, TNF-α has been targeted as a treatment of sepsis in many clinical trials. Although no trial has succeeded in showing an overall improvement using this therapy, several studies have identified populations and/or characteristics of these patients that may direct future trials.

The first large trial, NORASEPT, was done by Abraham et al. in 1995 and included 900 patients with sepsis or septic shock. The NORASEPT trial evaluated an anti-TNF-α monoclonal antibody and failed to show an overall mortality benefit. However, the subset of patients with septic shock showed a significant improvement in mortality 3 days after drug infusion. In following the patients further, the 28-day mortality continued to show a trend towards improvement but was no longer significant [23]. The INTERSEPT study, published in 1996, focused on evaluation of 420 patients with septic shock. This study showed more rapid reversal of shock and fewer patients with at least one organ failure in survivors who were treated with the anti-TNF-α monoclonal antibody as compared with the placebo group. However, this trial failed to show a difference in mortality [24]. This drug was tested in a third trial, NORASEPT II, which also failed to show an improvement in mortality [25].

A trial of an anti-TNF-α antibody fragment, afelimomab, was done by Reinhart et al. and published in 1996 that suggested a benefit of treatment in patients with baseline elevation of IL-6 [26]. Physiologically, this association is plausible as IL-6 levels are considered to be a surrogate for overall TNF-α activity due to the longer half-life of IL-6 compared to the rapidly cleared TNF-α. This hypothesis was tested in a prospective, randomized placebo-controlled trial, the RAMSES study of 446 patients with elevated IL-6 levels. It showed a nonsignificant trend towards improved survival in those treated with afelimomab [27]. A later study, the MONARCS trial, tested the same antibody fragment in 998 patients with elevated IL-6 levels and found a trend towards improved survival in treated patients as compared to placebo. The risk-adjusted reduction in mortality was 5.8%

and corresponded to a relative risk reduction for mortality of 11.9%. This study also found a greater reduction in IL-6 levels and multiorgan dysfunction score in those treated with afelimomab. The results are also encouraging because patients with higher IL-6 levels had significantly higher mortality rates in the placebo group than those with lower IL-6 levels. Thus, this showed that afelimomab had a greater effect in patients at higher risk of mortality [28]. In a similar investigation, cytofab, a preparation of polyclonal ovine anti-TNF Fab IgG fragments, was tested in a phase II placebo-controlled randomized clinical trial in 81 septic patients with shock or two organ dysfunctions. While this study did not show a difference in mortality, the investigators were able to show an increase in ventilator-free days, ICU-free days, and a decrease in serum and BAL levels of TNF-α and downstream effects on IL-6 in patients treated with CytoFab [29].

The persistent trends toward improved survival in the above studies are encouraging that some patients have the ability to benefit from immunotherapies. The difficulty lies in determining which patients are most likely to benefit. Are elevated IL-6 levels the correct marker for the selection of patients for use of anti-TNF-α therapy? Are elevated IL-6 levels a marker of worsening disease severity and, thus, improvement in this group of patients is due to their high severity of illness at presentation? Are IL-6 levels a reflection of timing of progression of sepsis? It is the hope that with further research, clinicians will be able to determine exactly which target population and at what point in their disease patients will benefit from a given treatment such as anti-TNF-α therapy.

With a similar mechanism of action, IL-1 is also a target of immunotherapies. This proinflammatory cytokine works together with TNF-α to propagate the hyperimmune response of sepsis. Macrophages and other cells naturally produce IL-1 receptor antagonist (IL-1ra) in response to IL-1, endotoxin, and various other microbial elements. The IL-1ra reversibly binds and competitively inhibits IL-1 receptors [30]. In 1994, Fisher et al. published a study evaluating the use of IL-1ra in the treatment of 893 patients with sepsis. This study failed to show an overall increase in survival in those treated as compared to placebo. However, retrospective and secondary analyses identified a trend of increased survival among patients with sepsis as well as an organ dysfunction and/or a predicted risk of mortality $\geq$ 24% [30]. Subsequently, Opal et al. published a trial in 1997 focusing on IL-1ra treatment in patients with severe sepsis and/or septic shock. Disappointingly, this study was halted when just over half of the proposed enrollment was completed and analysis revealed a low likelihood of showing a statistical difference in their primary endpoint, 28-day mortality. Secondary endpoints showed that those patients treated with IL-1ra displayed a nonsignificant trend towards improvement of organ dysfunction. The authors postulate that they may have had greater success if they were able to identify a more homogenous population. They were also concerned that their treatment was unable to maintain the necessary 100–10,000 fold excess of IL-1ra relative to IL-1 as it is known that stimulation of as few as 5% of the IL-1 receptors triggers an inflammatory response [31]. Perhaps further evaluation of this drug with the monitoring of levels to ensure complete blockage of the receptors or use of the drug in a more targeted population would provide a better chance for success.

5. Statins

There are many ways that statins have the ability to affect the immune response in sepsis and the exact mechanism of their action is unknown. Statins inhibit the reduction of hydroxymethyl-glutaryl-CoA to mevalonate which plays a role in synthesis of bile acids, some steroid hormones, and vitamin D. Statins inhibit various other pathways involved in pathophysiology of sepsis including inhibition of the production of cyclo-oxygenase-2 protein, biosynthesis of ubiquinone which functions in the electron transport chain of mitochondria, heme-A used in oxygen transport, and prenylation of small G proteins. It is likely that the alteration of the G-protein pathways has the most influential effect as this significantly alters inflammatory cell activation and protein production. Among other proteins, it is known to inhibit the production of subunits necessary for the GTP binding protein Rho. This inhibition has the downstream effect of production of a decreased amount of inflammatory cytokines such as IL-6 and IL-1. Furthermore, HMGCoA-reductase also induces caspase-dependent apoptosis in smooth muscle cells that may result in less inflammation due to avoidance of necrotic cell death [32].

Data from prospective, randomized-controlled trials evaluating the use of statin therapy in sepsis is lacking. However, multiple observational studies show encouraging effects. A large cohort study of more than 12,000 critically ill patients was published by Christensen et al. in 2010. Results showed that patients on statin therapy immediately prior to ICU admission had a decreased risk of mortality within 30 days and up to 1 year after ICU admission. Given the design of this study, the authors are unable to infer causation but the results stimulate excitement for further evaluation of the effects of statin use [33]. A large meta-analysis done by Bjorkhem-Bergman et al, published in 2010, evaluated the potential use of statin therapy in bacterial infection. It showed that patients on statin therapy seemed to have better outcomes including decreased mortality. However, when the 15 observational studies were adjusted for publication bias the association failed to reach statistical significance [34]. During that same year, Janda et al. focused the evaluation further when they published a meta-analysis evaluating statin therapy in severe infections and sepsis. This study included 20 trials, mostly cohort studies and one randomized-controlled trial that demonstrated a protective effect associated with statin use. The positive outcomes evaluated included 30-day mortality, in-hospital mortality, pneumonia-related mortality, bacteremia-related mortality, sepsis-related mortality, and mixed infection related morality. Again, this study was limited due to the inclusion of mostly cohort studies and significant heterogeneity of trials [35]. The one randomized controlled trial in this data set was completed by Tseng et al. and included 80 patients with aneurysmal subarachnoid hemorrhages. While this study

did show an improvement in sepsis-associated mortality, it cautioned that this finding was a secondary outcome [36]. Due to the promising effects of statins, both based on physiologic knowledge and on the current observational data, phase II and phase III studies are currently in progress to evaluate the role of statins in the treatment of sepsis.

6. Inhibition of the Coagulation Cascade

The extreme activation of the inflammatory system in severe sepsis is accompanied by a potentially equal stimulation of the coagulation system. From an adaptive perspective, this interaction is logical as the activation of the coagulation system can be envisioned as an effort to isolate the infection with the goal of limiting its spread throughout the body. However, in the process of severe sepsis, this activation results in a futile and, likely, counterproductive endeavor as the infection has already spread throughout the bloodstream and the coagulation system activation results in diffuse microvascular thrombi with wide spread endothelial damage and organ failure.

Various steps of the coagulation pathway have been targeted in the treatment of sepsis. Tissue factor (TF), a cell surface receptor whose expression by endothelial cells and monocytes occurs in the presence of inflammatory mediators, acts to initiate the extrinsic coagulation pathway. A TF inhibitor was tested in the Phase III trial, OPTIMIST, evaluating its use in 1,754 patients with severe sepsis and this trial failed to show an improvement in mortality. More concerning, it showed a trend towards harm in those treated concurrently with heparin [37]. Similarly, antithrombin III (AT III), an anticoagulant, was the subject of sepsis therapy as well due to the finding of decreased AT III levels in severe sepsis and the hypothesis that this deficiency contributes to the hypercoagulation pathophysiology in sepsis. Multiple small studies published in the 1990s showed promising results. However, in a phase III trial of 2,314 septic patients, they were unable to show a difference in overall mortality. However, in subgroup analysis, patients not treated concomitantly with heparin showed a significant decrease in mortality at 90 days while those treated with heparin showed a significantly increased risk of bleeding [38]. Future investigation of AT III as a treatment for sepsis will need to carefully select their target population to ensure minimal risks for bleeding.

To date, the only drug that has been approved for the treatment of severe sepsis is recombinant human activated protein C (rhaPC). It was investigated due to its anti-apoptotic, anti-inflammatory, and anticoagulant effects. It acts via inhibition of factors Va and VIIIa which results in the prevention of thrombin generation. Downstream, this decreases inflammation by reducing mast cell degranulation, platelet activation, and neutrophil recruitment [5, 39]. The PROWESS trial, published in 2001 spurred great excitement due to its absolute reduction in 28-day mortality by 6.1% in septic patients treated with rhaPC and it was subsequently approved for use in the most severely ill septic patients with APACHE scores greater than 25 as this subgroup seemed to derive the most benefit from treatment [39]. Unfortunately, these results were not replicated in the PROWESS-SHOCK study and the treatment was voluntarily removed from the market by the manufacturer [40]. The use of rhaPC is not recommended for use in children based on a study published in 2007 that evaluated 477 septic children and failed to show an improvement in mortality [41].

Thrombomodulin (TM), another naturally occurring pathway in the coagulation system, is currently being targeted in the treatment of sepsis. TM, produced by endothelial cells, acts upstream in the activated protein C pathway to sensitize the thrombin receptor leading to activation of protein C [42]. It has been shown that the serum concentration of TM parallels the severity of coagulopathy and organ failure in sepsis and decreases as DIC and ARDS improves [43]. A control study of 20 patients with severe sepsis-induced DIC treated with rhTM compared to 45 historical controls showed improved 28-day mortality and improved organ dysfunction in those treated with rhTM [42]. Ongoing phase II studies are in progress to evaluate the efficacy of rhTM [5].

7. Immunostimulation

Due to the recognition that sepsis is characterized by a combination of hyperimmune response and relative immunoparalysis, further investigations have pursued immunostimulatory strategies. A controversial and widely studied therapy is treatment with the use of pooled serum polyclonal immunoglobulin preparations, IVIG. Although the exact mechanism remains in question, it is thought that the immunoglobulins coat bacteria, which improves phagocytosis and enhances neutralization and opsonization causing inactivation of bacterial endotoxins and exotoxins. Furthermore, it is hypothesized that the treatment alters the release of cytokines and cytokine antagonists by endotoxin and interacts with the complement cascade causing an improved immune response in sepsis [10]. Further supporting this strategy is a recent study which evaluated 62 adult septic patients and revealed decreased levels of immunoglobulins particularly IgG and IgM early in sepsis as compared to age-matched controls. This was followed by normalization of levels after 7 days in the majority of patients. Decreased level of immunoglobulins was associated with decreased levels of plasma proteins but was not associated with a difference in mortality [44].

In 2007 and 2008, three meta-analyses were published that evaluated the efficacy of polyclonal IVIG in adult patients with sepsis. All three concluded that this therapy improved survival but, due to small study sizes, heterogeneity, and methodologic limitations of the individual studies, the three authors recommended large randomized, controlled trials to verify therapeutic efficacy [45–47]. A subsequent Cochrane review published in 2010 evaluated 17 trials of polyclonal IVIG in adult patients with sepsis. This review was in agreement with the prior meta-analyses and showed a reduction in less than 30-day mortality in treated patients. However, the authors recommended

cautious interpretation of their findings as the majority of studies had a small sample size and there was concern for poor methodologic quality. Furthermore, when the trials were restricted to those with low risk of bias, no reduction in mortality was shown and the studies that evaluated long-term mortality (greater than 60 days) did not show an effect. The Cochrane review went on to specify their agreement with the Kreymann et al. meta-analysis findings that the IgM-enriched formulation of immunoglobulin is also beneficial and even trended toward a greater effectiveness in the treatment of sepsis [10, 45]. The authors conclude that polyclonal immunoglobulins appear to be beneficial as adjuvant therapy for sepsis but recommend large, multicenter studies for confirmation [10].

The pediatric population stands to reap greater benefit from IVIG due to the immaturity of B-cells in patients less than 5 years old. In 2005, a prospective case-controlled trial of 100 pediatric patients showed a significant improvement in length of stay, development of complications, and mortality in septic pediatric patients 1 month–24 months old treated with IVIG [48]. Based on the findings of this study, the Surviving Sepsis Guidelines recommend consideration of IVIG treatment of pediatric patients with severe sepsis. However, this recommendation is supported only by weak evidence due to low trial quality [4]. IVIG in the neonatal population is equally as controversial as the Cochrane review found no reduction in mortality in septic neonates treated with IVIG while the Surviving Sepsis Guidelines cite that there is evidence to support improved mortality in neonates treated with IVIG [4, 10]. A study published by Brocklehurst et al. in 2011, after the publication of the Surviving Sepsis Guidelines and Alejandria's Cochrane review, evaluated over 3,000 neonates with sepsis and found no difference in the primary outcomes of mortality or major disability up to two years of age [49].

Other immunostimulatory strategies include cytokine stimulation with granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), and IFN-gamma. The hypothesized mechanism of these therapies in nonneutropenic patients is stimulation of bactericidal activity via increased leukocytosis and increased activity of granulocytes. Bo et al. published a meta-analysis of 21 randomized-controlled trials evaluating G-CSF and GM-CSF in the treatment of sepsis. This evaluation of a combined 2,380 septic patients showed no change in mortality but did show a positive effect of this therapy on the rate of reversal of infection. They found no difference in adverse events between the groups and recommended further studies to evaluate the efficacy of this therapy [50]. It is important to remember that G-CSF and GM-CSF differ in that GM-CSF has additional monocytic and macrocytic stimulatory affects, inducing monocytic cytokine expression and antigen presentation via increased expression mHLA-DR theoretically resulting in improved adaptive immunity [51]. In the meta-analysis by Bo et al., the data evaluating these two therapies were combined despite their differing mechanisms of action. Furthermore, the studies differed significantly on dose as well as on the route of administration and failed to stratify the patients based on their immunologic state. Given the immune-stimulatory mechanism of this therapy, it would be important to know if the patients being studied are in the hyper or hypoimmune phase of sepsis as this may affect the drug's efficacy. Furthermore, new data has shown that it is possible to track the efficacy of immunostimulatory therapies by measurement of mHLA-DR expression on monocytes which is decreased in patients with sepsis-associated immune cell dysfunction [51, 52]. Meisel et al. recently showed that patients with sepsis-associated immunosuppression, defined as low monocytic HLA-DR expression, who were treated with GM-CSF had improvement of monocytic HLA-DR expression when compared to placebo patients. Although this trial included only 38 patients, they were able to show shorter duration of mechanical ventilation as well as shorter ICU and hospital lengths of stay [52].

IFN-gamma has shown similar ability to restore monocytic HLA-DR expression in septic patients with evidence of monocyte deactivation. A small study done by Docke et al. showed that IFN-gamma treatment in septic patients with low monocytic HLA-DR expression resulted in restoration of monocyte function as measured by improved TNF-α secretion resulting in clearance of sepsis in 8 of 9 patients [53]. The results of these studies are encouraging that immunostimulation may be an effective way to treat the subset of septic patients who are in the immunoparalysis phase of their disease.

8. Directions for Future Research

Given the incredible number of patients affected with and dying due to sepsis, it is disappointing that the proven treatments of this disease have not expanded beyond that of fluids, vasopressors, and source control. To date, the knowledge gained form laboratory research and clinical trials has better defined the pathophysiology of the disease and the population of patients we are treating. However, rather than clearly providing new treatments, it has left us with more questions than answers. Despite the disappointing results of clinical trials which have been unable to find a universal treatment effective in all patients, some of the trials have shown promise in specific groups of patients. For example, HA-1A may improve outcomes in patients with gram-negative infections and anti-TNF-α therapies may effectively treat patients with elevated IL-6 levels despite these individual therapies' inability to treat all-comers with sepsis. TLR-4 inhibitors such as TAK-242 may have greater effect when used earlier in the course of the illness or when used in the most severely ill cohort of patients. Immunostimulatory medications may improve patients in the hypoimmune phase of illness as identified by low monocytic HLA-DR expression or another yet-to-be identified marker of the immunoparalysis phase. Therapies with more hazardous side effect profiles such as steroids or activated protein C may prove to be efficacious in the most severely ill patients or those with greater coagulopathy, where the risks of the disease progression outweigh the risks of the therapy. Even better, we may develop a test or clinical profile that will allow us to better identify the patients most likely

to benefit from the specific therapy or provide the subset of patients at minimal risk of an adverse event. It is also conceivable that concurrent therapy will help dictate the best treatment option. For example, a study of AT III restricted to patients without concomitant anticoagulation therapy may show that it can serve as a beneficial treatment for sepsis.

Further research may help to clarify the role of genetics in improving individualization of therapies as well. For more than ten years, genetic studies have evaluated links between polymorphisms of the major histocompatibility complex genes on chromosome 6 and human leukocyte antigen genes to the body's response to infection [54]. While it has yet to translate into clinically significant data, genetic studies have identified various polymorphisms associated with an increased risk of infection. Many of these polymorphisms cause alterations in the body's immune response. For example, one such polymorphism lies in the promoter region of TNF-α. A second polymorphism causes alterations of the two well-studied toll-like receptors, TLR2 and TLR4, which provide the innate immune system with the ability to recognize and respond to gram-positive and gram-negative bacteria. Further studies have evaluated expression profiling via measurement of mRNA. These studies and the help of computer technology have led to identification of subclasses of children with septic shock based on similar patterns of gene expression [55]. With an improved ability to link genetics to a patient's specific disease process and, further, to therapeutic response, a more customized approach to therapy could be achieved both by directing specific therapies as well as by creating more homogenous populations of study patients with an improved ability to show efficacy in clinical trials. To add a greater degree of complexity, perhaps the use of multiple therapies in order to attack the disease process from different approaches will prove to be the best customization of therapy.

It is the above variables including but not limited to the infecting organism, phase of illness, severity of illness, host's inflammatory response, and genotype that make this disease process exceedingly difficult to combat. Ideally, it would be possible to construct homogenous septic patient populations in order to appropriately test various therapies in subgroups of patients. However, no single ICU has the ability to generate the numbers necessary in order to evaluate and prove efficacy of these personalized treatment strategies. On the other hand, modern computer technology allows us to search and evaluate large sums of data. Not only does this give us the opportunity to pool data from multiple sites in an efficient manner but also it allows us to search this data in a very sophisticated way. In doing so, it may be possible to identify patterns based on clinical symptoms, laboratory studies, comorbidities, genomic information, response to therapy, biomarkers, and so forth, that will enable us to form groupings of patients and monitor their response to treatment options carefully selected based on our knowledge of the mechanism of action of the therapy and understanding of the patient's disease process.

In 2004, Science Applications International Corporation and Merck Capital Ventures studied the advances in technology and the factors influencing their adoption rates in the use of clinical trial development. The study gathered information by reviewing industry-sponsored research, performing a literature research and interviewing those with significant experience in clinical development process, especially in business processes and information technology (IT). Their study showed a significant resistance of moving away from paper-based system and to new IT. In contrast, it also showed an increasing acceptance of IT in the face of regulatory pressures to improve adverse event reporting and improve the success of submissions. Furthermore, they found that process change is the key to improving the core function of the system and that the addition of technology alone, without alteration of existing processes, is not sufficient. Their belief is that IT can benefit clinical research in the ways of improved cycle time, data quality, and cost effectiveness. Current clinical trial structures are fraught with incompatible systems, complicated data entry formats and challenging organization for data searching as well as exchanging of information. This study sites outcomes such as centralization of data, advanced data mining capabilities, vocabulary standards, and cross-trial data pooling that could be achieved by adoption of new IT pathways to advance the field of clinical trials [56]. In 2004, the FDA presented the report, *Innovation or Stagnation, Challenge and Opportunity on the Critical Path to New Medical Products*, which detailed its concerns regarding the field of drug development. It described a 50% decline in new product submissions to the FDA over the previous 10 years in spite of a 250% increase in research and development expenditures. Their analysis found that 50% of drugs that showed promise in phase II trials went on to fail in phase III studies and that only one in ten drugs that undergo clinical testing eventually obtain FDA approval. Furthermore, it takes an average of 15 years and nearly a billion dollars in research and development to reach the clinical market. They cited the major component of the inefficiency in drug development as the lack of modern methods for drug testing stating, "Often, developers are forced to use the tools of the last century to evaluate this century's advances." [57]. The efforts to improve the field of clinical research to the level of technology incorporated in other areas of business will be well worth the investment. The outcomes will benefit the patients we care for by providing an improved understanding of the methods to combat sepsis and the ability to deliver of the most up-to-date treatments.

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