Short Communication

Novel mechanism of immunosuppression by influenza virus haemagglutinin: selective suppression of interleukin 12 p35 transcription in murine bone marrow-derived dendritic cells


1,3Immunovirology Laboratory, Institute of Immunology and Biology Department and Mucosal Immunology Laboratory, Institute of Immunology, National University of Ireland, Maynooth, Co. Kildare, Ireland

2National Institute for Biological Standards and Controls, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3QG, UK

4Immune Regulation Research Group, Biochemistry Department, Trinity College Dublin, Ireland

Infection with influenza virus strongly predisposes an individual to bacterial superinfection, which is often the significant cause of morbidity and mortality during influenza epidemics. Little is known about the immunomodulating properties of the virus that lead to this phenomenon, but the effect of the viral components on the development of immune dendritic cells (DCs) may prove vital. In this study, activation of and cytokine secretion by bacterial lipopolysaccharide (LPS)-stimulated bone marrow-derived dendritic cells (BMDCs) following treatment with the influenza virus major antigen haemagglutinin (HA) were examined. HA selectively inhibits the release of LPS-induced interleukin 12 (IL12) p70, which is independent of IL10 secretion.

Suppression occurs at the transcriptional level, with selective inhibition of p35- and not p40-subunit mRNA expression. The downregulation of IL12 p70 by influenza HA is a novel and unexplored pathway that may be relevant in the predisposition to bacterial superinfection associated with influenza virus infections.

Influenza virus causes yearly epidemics of respiratory illness worldwide and is one of the most prevalent causes of hospitalization for acute respiratory disease (Howlett, 2004; Reichert et al., 2004; Thompson et al., 2004). Bacterial superinfections are a major cause of morbidity and mortality in influenza virus infections (Hament et al., 1999; Okamoto et al., 2003; Seki et al., 2004; Takase et al., 1999) and, whilst the host appears to be immunosuppressed during influenza virus infection, the mechanisms underlying this predisposition to bacterial infection remain poorly defined (Beadling & Slifka, 2004; Glezen, 2004; Hament et al., 1999; Hussell & Williams, 2004; LeVine et al., 2001; Okamoto et al., 2003; Seki et al., 2004). Studies have suggested that physical damage to respiratory cells as a result of virus infection may lead to opportunistic adherence of bacteria, and enhanced bacterial adherence via virus surface receptors has been reported for respiratory cells infected with influenza A virus (Hament et al., 1999; Sanford & Ramsay, 1987). However, there is also evidence that influenza virus downregulates neutrophil function and accelerates neutrophil apoptosis, suggesting a more physiological role for influenza virus in the transition from asymptomatic carrier to invasive bacterial disease (Colamussi et al., 1999; Hartshorn et al., 1995). In order to examine further the role of immunomodulation in the increased susceptibility to bacterial superinfection during influenza virus infection, we investigated whether haemagglutinin (HA), the surface glycoprotein of influenza virus, could modulate dendritic-cell (DC) maturation and cytokine production in response to bacterial lipopolysaccharide (LPS).

Bone marrow-derived immature DCs (BMDCs) were prepared by culturing bone-marrow cells obtained from the femur and tibia of 6–8-week-old BALB/c mice (Harlan UK) in RPMI 1640 medium and 10 % fetal calf serum (FCS) supplemented with 5–10 % supernatant from a granulocyte–macrophage colony-stimulating factor (GM-CSF)-expressing cell line (provided by Nathalie Winter, Institut Pasteur, Paris, France, with permission of David Gray, University of Edinburgh, Edinburgh, UK). Cells were washed and fed with fresh RPMI/10 % FCS containing 10 % GM-CSF cell supernatant every 3 days for a period of 8 days. Flow cytometry revealed 80–85 % CD11c+ cells and expression of B7.1, B7.2, major histocompatibility complex (MHC) class II and CD40 markers, which were upregulated
following treatment with LPS. BMDCs (10^6 ml^-1) were cultured with HA [from influenza virus strain A/Panama/2007/99 (H3N2)] (0, 2 or 4 μg ml^-1), LPS (2 μg ml^-1) or a combination of LPS (2 μg ml^-1) and HA (2 or 4 μg ml^-1). Cell supernatants were collected at 24 h and the levels of cytokines and chemokines were determined by ELISA. HA was obtained from the National Institute of Biological Standards and Controls (NIBSC), Herts, UK. HA antigen was extracted from purified virus by treatment with bromelain and purified by sedimentation on sucrose gradients (Brand & Skehel, 1972). We demonstrate that HA from influenza virus selectively inhibits the release of the cytokine interleukin 12 (IL12) (bioactive) by bacterial LPS-stimulated BMDCs in a dose-dependent manner (Fig. 1a). Pre-incubation with HA did not alter the suppressive effect (Fig. 1b), which was specific for IL12, exerting no effect on the LPS-induced production of other pro-inflammatory cytokines [IL1β, tumour necrosis factor alpha (TNF-α)] or IL6 or chemokines [monocyte chemotractant protein (MCP)-1, regulated on activation, normal T cell-expressed and secreted (Rantes), macrophage inflammatory protein (MIP)] by BMDCs (Fig. 1c). LPS-induced IL12 production could be restored by pre-incubation with sheep anti-HA (NIBSC, Herts, UK), which did not induce IL12 production alone (Fig. 2a). HA did not induce necrosis or apoptosis of these cells, as demonstrated by ethidium bromide/acridine orange or annexin V staining, respectively, and HA-treated BMDCs underwent normal maturation processes in response to LPS, as demonstrated by upregulation of B7.1, B7.2, MHC class II and CD40 expression (data not shown).

IL12 is a 70 kDa pro-inflammatory cytokine that is produced by phagocytic cells and antigen-presenting cells, in particular macrophages and DCs, as a result of non-antigen-specific stimulation with bacteria, bacterial products such as LPS and intracellular parasites (O’Garra, 1998; Trinchieri, 1998). Initially, it was suggested that IL12 was a prerequisite

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**Fig. 1.** HA from influenza virus selectively downregulates LPS-induced IL12 p70 production by murine BMDCs. (a) IL12 p70 levels following treatment with medium (0 μg HA ml^-1), HA1 (2 μg ml^-1), HA2 (4 μg ml^-1), LPS (2 μg ml^-1), LPS and HA1 or LPS and HA2. (b) IL12 p70 levels following treatment with medium, HA2, LPS, and HA2 or HA2 added 1 h before treatment with LPS. (c) Levels of cytokines (IL12 p40, TNF-α, IL1β and IL6) and chemokines (MCP-1, MIP-1α, MIP-1β and RANTES) produced by BMDCs incubated with medium, HA2, LPS or LPS and HA2. Results are expressed as means ± SEM and were compared by using Student’s t-test. P values < 0.05 were considered statistically significant. Data are representative of at least four experiments.
in the promotion of cellular responses and the resolution of the majority of intracellular infections (Magram et al., 1996; O’Garra, 1998; Trinchieri, 1998). Recent studies, however, imply that IL12 is less essential in Th1 responses to viral rather than bacterial infections (Oxenius et al., 1999; Schijns et al., 1998; Xing et al., 2001). Our finding that influenza virus HA downregulates DC bioactive IL12 may shed some light on the predisposition to bacterial super-infection that is so prevalent with this disease.

Studies have demonstrated a role for autocrine production of IL10 in the suppression of IL12 responses in vivo and in vitro (Aste-Amezaga et al., 1998; McGuirk et al., 2002). To further elucidate the mechanism underpinning IL12 down-regulation by influenza virus HA, we explored the possibility that IL10 may mediate the effect. We demonstrate that HA does not induce IL10 alone, nor does it synergize with LPS to produce elevated levels of this cytokine. Furthermore, pre-incubation with an anti-IL10 antibody did not revert the suppressive effect of HA on LPS-induced IL12 production by BMDCs (Fig. 2b). It has also been demonstrated that, during viral infections, early type 1 alpha and beta interferons (IFN-α/β) downregulate IL12 p70 production (Cousens et al., 1997). However, the lack of effect on other cytokines, particularly IL12 p40, which is negatively regulated by IL10 (Aste-Amezaga et al., 1998; McGuirk et al., 2002), and the inability to block the effect with anti-IL10 antibodies suggest that a cytokine intermediate is not involved in the downregulation of LPS-induced IL12 production by HA. Similar to our finding with anti-IL10, we could not restore IL12 p70 production by pre-incubation with an anti-IFN-α/β antibody (data not shown).

Bioactive IL12 differs from other pro-inflammatory cytokines in that it is a heterodimer, comprising 35 kDa (p35) and 40 kDa (p40) polypeptides encoded by separate

![Fig. 2. HA downregulation of IL12 p70 production by BMDCs can be reverted by anti-HA antibodies, is not restored by neutralization of IL10 and is applied at the level of IL12 p35 mRNA transcription. (a) IL12 p70 levels in BMDC supernatants at 24 h following treatment with medium, anti-HA (15 μg ml⁻¹), HA2 (4 μg ml⁻¹), LPS (2 μg ml⁻¹) or LPS (2 μg ml⁻¹), HA (4 μg ml⁻¹) and anti-HA (15 μg ml⁻¹). (b) Murine BMDC IL12 p70 and IL10 levels 24 h post-stimulation with medium, HA2, LPS or HA2, with or without anti-IL10 (10 μg ml⁻¹). (c) RT-PCR of IL12 p35 and IL12 p40 at 2, 4, 6 and 24 h post-treatment of BMDCs with medium (lane 1), HA2 (4 μg ml⁻¹; lane 2), LPS (2 μg ml⁻¹; lane 3) or LPS and HA2 (2 and 4 μg ml⁻¹, respectively; lane 4). +, Positive control (BMDCs positive for p35 expression); −, negative control (BMDCs negative for p35 expression). Where applicable, results are expressed as means ± SEM and were compared by using Student’s t-test. P values <0.05 were considered statistically significant. Data are representative of three experiments.](http://vir.sgmjournals.org)
genes (O'Garra, 1998; Trinchieri, 1998). Although both subunits must be co-expressed within the same cells to generate the bioactive form, evidence has suggested that expression of each subunit is regulated independently (Murphy et al., 1995). It has been suggested that p40 expression controls the level of bioactive IL12, as this subunit is highly regulated, whereas p35 is expressed ubiquitously (Snijders et al., 1996). However, in our study, the levels of p40 protein remained elevated, despite the downregulation of the heterodimer p70. These findings lend weight to other reports that attest to the regulation of p35 and suggest that p35 is the limiting factor in bioactive IL12 production, as the level of p40 exceeds that of p35 (Gillessen et al., 1995; Heinzel et al., 1997). In addition, the finding that p40 is frequently produced in excess as a homodimer ([p40]2) that can bind to the IL12 receptor and antagonize IL12-mediated function suggests that the biological activity of IL12 may be determined in part by the ratio of p40 to p70 (Gillessen et al., 1995; Heinzel et al., 1997). This, in turn, would indicate that p35 levels are likely to be an important limiting factor in determining bioactive IL12 production. In the light of earlier reports regarding p40 expression, the molecular mechanisms of its regulation have been well-characterized (Gillessen et al., 1995; Heinzel et al., 1997; Murphy et al., 1995). In contrast, the mechanisms underlying p35 expression in health and disease remain largely ill-defined.

To further examine the effects of HA on the expression of both subunits of the heterodimer, the level of p40 and p35 mRNA expression was examined by RT-PCR. For RT-PCR studies, total RNA was harvested from cells at 2, 4, 6 and 24 h and the level of expression of IL12 p40 and IL12 p35 mRNA was determined by RT-PCR, using published primer sequences. The presence of HA in LPS-stimulated cultures of BMDCs did not alter p40 expression, but completely abrogated LPS-induced p35 mRNA expression up to 4 h post-treatment (Fig. 2c). p35 expression was restored at 24 h in these samples, although protein levels still demonstrated significant reduction in IL12 p70 at 24 h. This was possibly due to the delay in generating protein from mRNA and could be considered to be consistent with inhibition of mRNA transcription for at least one-third of the total treatment period. This selective inhibition of p35 gene expression is a novel and unexplored pathway to downregulate IL12 responses. These findings shed light on the control of bioactive IL12 p70 expression in DCs. The speed of inhibition of p35 expression by HA argues against the possibility of regulation by autocrine expression of cytokines, but suggests that HA interferes directly with the pathway of p35 gene activation. Furthermore, it rules out possible interference at the level of receptor binding, as intracellular signalling pathways for p40 and expression of other cytokines are unimpaired. Reports have suggested that p35 protein expression is regulated at both the transcriptional and translational levels (Grumont et al., 2001; Murphy et al., 1995). Although translational control cannot be ruled out clearly in this system, the complete abrogation (at 2 and 4 h) of p35 mRNA by HA is due to transcriptional control. A recent report has shown that the Rel/NF-kB signalling pathway is required for the induction of IL12 in activated CD8+ splenic DCs (Grumont et al., 2001). Impaired expression of IL12 p70, but not p40 or (p40), in c-rel−/−CD8+ DCs is due to an inability to upregulate p35 transcription, which is c-rel-dependent (Grumont et al., 2001). The similarity in cytokine-expression profiles between LPS-stimulated c-rel−/− DCs and LPS-stimulated, HA-treated BMDCs leads us to speculate that HA may target c-Rel-dependent transcription. Furthermore, in comparison with c-Rel−/− DC studies, HA does not interfere with or disrupt normal DC maturation, whilst IL12 expression (p70, p35) is impaired (Grumont et al., 2001). To establish whether HA exerts its suppressive effect in vivo as well as in vitro, groups of four BALB/c mice were injected intravenously with HA (10 μg per mouse) and LPS (1 μg per mouse) alone or in combination and serum concentrations of IL12 and IFN-γ were assessed by ELISA at 6 h post-injection. Mice were rested for 1 h between each dose. Consistent with DC studies, HA significantly downregulated LPS-induced IL12 p70 production in mice, which was accompanied by a reduction in IFN-γ levels (Fig. 3).
The finding that HA from influenza virus can modulate in vitro and in vivo responses to bacterial LPS may elucidate the mechanism underlying the predisposition to bacterial superinfection that is associated with influenza virus infection. p40−/− mice demonstrate increased bacterial infection compared with p35−/− mice, possibly due to the fact that p40 may dock with another subunit, p19, forming IL23, a cytokine that has properties similar to those of IL12 (Oppmann et al., 2000). However, p35-knockout animals also demonstrate increased susceptibility to infection and delayed clearance of bacteria (Lehmann et al., 2001; Oppmann et al., 2000).

To our knowledge, no other study has demonstrated immunosuppressive properties for HA, the major surface antigen of influenza virus. Whilst studies have defined a clear role for IL12 in the development of Th1 responses to bacterial infections, such a monopoly for IL12 in the induction of the Th1 response to viral infection is less definitive. Several recent studies have demonstrated that mice lacking IL12 can develop polarized Th1 cells during viral infection (Oxenius et al., 1999; Schijns et al., 1998). In addition, it has been reported that influenza virus infection fails to induce p35 or p40 mRNA expression in human macrophages (Pirhonen et al., 2002). We also observe identical Th1 responses from IL12-knockout mice compared with wild-type controls, following challenge with live influenza virus (C. M. Noone & P. A. Johnson, unpublished observations). This begs the question as to the likely advantage that the inhibitory effect of HA may have for virus survival. Increasingly, studies have revealed the enormous redundancy that is built into the immune system and that, whilst a virus may subvert one pathway, which may historically have prolonged its survival, the host, likewise, may have evolved other means to eliminate the infecting virus. An alternative and possibly more likely explanation is that the p35 pathway is also involved in some as-yet- unidentified pathway of virus clearance and that, whilst p35 expression may be redundant in antiviral responses, the p35 pathway may not be. Studies carried out in IL12-knockout mice have traditionally targeted the p40 pathway, so the influence of the pathway that leads to p35 expression on antiviral immunity remains to be explored. Thus, inhibition of this pathway may confer some selective advantage on the virus, independent of type 2 IFN production. Influenza is a successful pathogen: even beyond its ability to generate new antigenic types, it possesses other immune-subversive tactics, such as the inhibition of the type 1 IFN pathway in myeloid-derived DCs by the non-structural protein of the virus (Marcus et al., 2005). It is plausible that other viral proteins may also be involved in immune subversion/modulation.

These results suggest that, for influenza virus infection, bioactive IL12 p70 is not an absolute requirement for a polarized Th1 response during infection; however, downregulation of this cytokine may render the host more susceptible to bacterial colonization. We therefore propose that the mechanisms underlying bacterial superinfection in viral respiratory disease are not simply a consequence of increased bacterial adherence, but involve more complicated physiological mechanisms relating to the immunomodulating properties of key viral antigens. We hypothesize that virus components alter the course of critical immune responses to bacterial invasion, rendering the host more susceptible to infection. Our finding that HA from influenza virus modulates IL12 production by BMDCs represents a novel and unexplored pathway by which influenza virus may render the host more susceptible to bacterial infections.

References

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