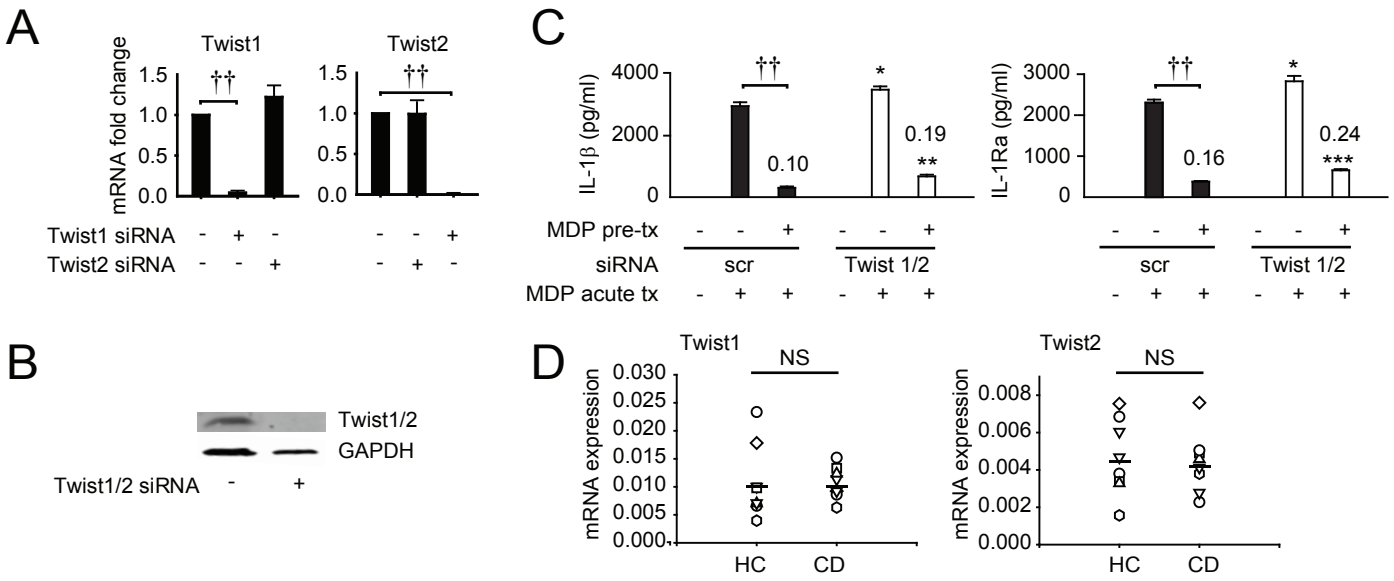
# Supplemental Figure 1



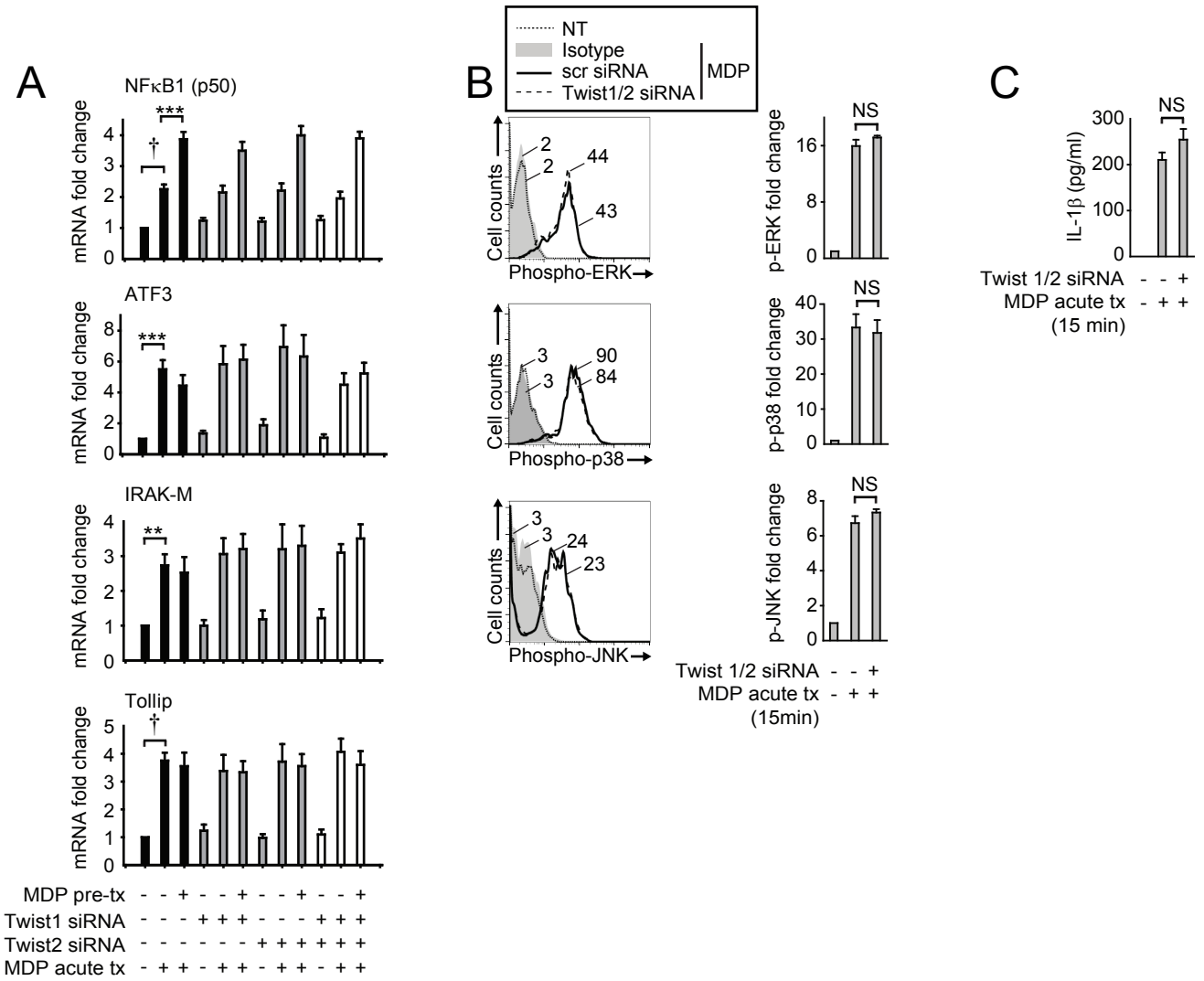

## E Description of Crohn's Disease Patients

| Diagnosis[1] | Age (yrs) | Gender | Dz[2] duration (yrs) | Location | Medications[3] | NOD2 CD risk SNP[4] |
|---|---|---|---|---|---|---|
| CD | 30 | Female | 16 | Colonic | AZA, Abx | WT/WT |
| CD | 40 | Female | 4 | Ileocolonic | Anti-TNF | WT/WT |
| CD | 46 | Male | 32 | Ileocolonic | AZA, Anti-TNF | WT/WT |
| CD | 52 | Male | 6 | Ileocolonic | 5-ASA | WT/WT |
| CD | 28 | Male | 8 | Ileocolonic | AZA, Anti-TNF | WT/WT |
| CD | 28 | Female | 10 | Ileocolonic, Perianal | Anti-TNF | WT/WT |
| CD | 38 | Male | 17 | Ileocolonic | Anti-TNF | WT/WT |
| CD | 22 | Female | 1 | Ileocolonic | | WT/WT |
| CD | 56 | Male | 44 | Ileocolonic | Anti-TNF, Abx | Cins/Cins |
| CD | 23 | Male | 4 | Ileocolonic, Perianal | Anti-TNF | Gly908Arg/Gly908Arg |
| CD | 30 | Female | 19 | Ileocolonic | 5-ASA | Cins/Gly908Arg |

[1]CD, Crohn's disease (European Ancestry patients); [2]Dz, disease.
[3]AZA, azathioprine; Abx, antibiotics; anti-TNF, anti-tumor necrosis factor-α; 5-ASA, 5-aminosalicylic acid
[4]CD-associated NOD2 genotype: SNP, single nucleotide polymorphism; WT, wild-type; Cins, Leu1007fsinsC

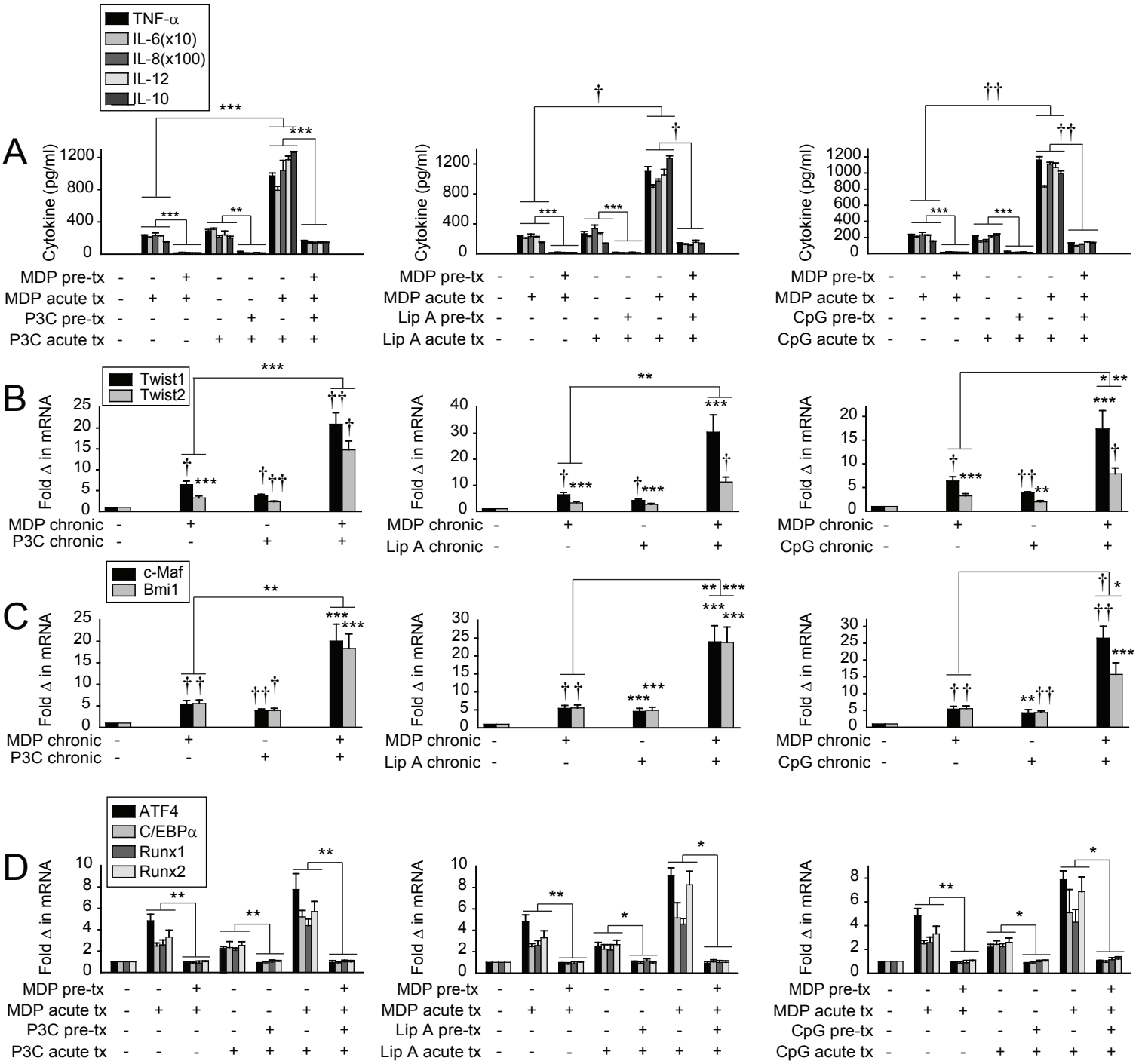
**Supplemental Figure 1. Twist1 and Twist2 siRNA effectively decrease Twist mRNA and protein expression.** **(A)** Human MDMs (n=8) were transfected with scrambled, Twist1 or Twist2 siRNA, and 48h later MDMs were assessed for Twist1 or Twist2 RNA expression relative to scrambled siRNA-transfected cells + SEM. **(B)** Human MDMs were pre-treated with 100μg/ml MDP for 24h, then transfected with scrambled, or a combination of Twist1 and Twist2 siRNA, and 24h later (total 48h MDP pre-treatment), MDMs were treated with 100μg/ml MDP for an additional 8h. Western blot was conducted with an antibody detecting both Twist1 and Twist2. Representative Western blot from 1 of 4 individuals. GAPDH is used as a loading control. **(C)** Human MDMs were left untreated (for acute) or pre-treated with 100μg/ml MDP for 24h, then transfected with scrambled, or combined Twist1 and Twist2 siRNA, and 24h later (total 48h MDP pre-treatment), MDMs were treated with 100μg/ml MDP for an additional 24h (acute). Supernatants were examined for cytokines. Mean + SEM for n=4. Numbers on the bars are the ratios of cytokine secretion upon MDP treatment of pre-treated versus non-pretreated MDMs. **(D)** MDP pre-treated/treated MDMs from WT NOD2 healthy controls (HC; n=8) and Crohn's disease (CD) patients (n=8) shown in Figure 2D were assessed for Twist1 and Twist2 mRNA expression by CT values normalized to GAPDH and represented as a linear scale. Each symbol represents an individual. Horizontal line represents the mean. **(E)** Phenotype/genotype characteristics for Crohn's disease patients studied in (D) and Figure 2D. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ††, $p<1\times10^{-5}$.

# Supplemental Figure 2



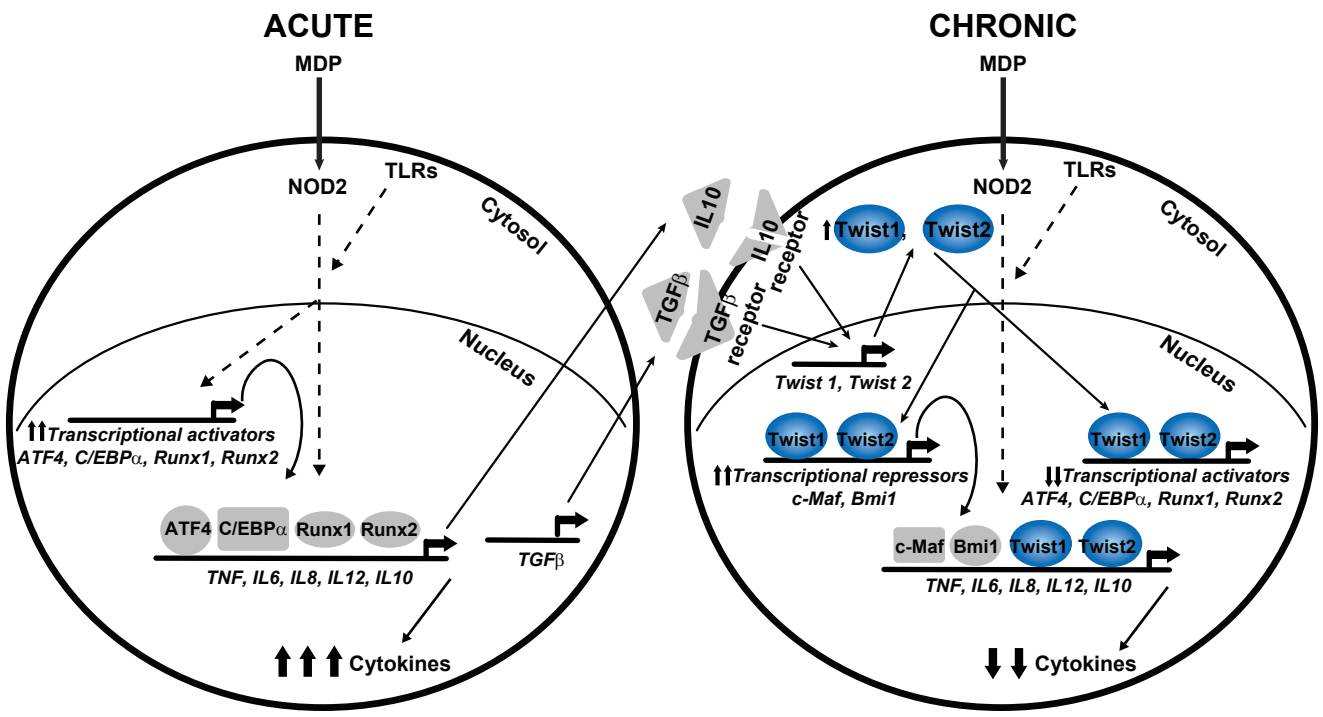

**Supplemental Figure 2. Twist1 and Twist2 do not contribute to upregulation of the intracellular inhibitory molecules NFκB1, ATF3, IRAK-M or Tollip, the activation of MAPK pathways or the early secretion of IL-1β.** **(A)** MDMs (n=8) were left untreated (for acute) or pre-treated with 100μg/ml MDP for 24h, then transfected with scrambled, Twist1 or Twist2 siRNA, alone or in combination, and 24h later (total 48h after MDP pre-treatment), MDMs were treated with 100μg/ml MDP for 4h (acute) and assessed for NFκB1, ATF3, IRAK-M and Tollip mRNA expression. Fold mRNA expression normalized to untreated, scrambled siRNA-transfected MDMs + SEM. **(B)** MDMs (n=8) were transfected with scrambled or combined Twist1 or Twist2 siRNA. Cells were then treated with 100μg/ml MDP for 15 min. (*Left*): Representative flow cytometry with MFI values for phospho-kinases. (*Right*): Summary graphs for phospho-kinase induction + SEM. **(C)** MDMs (n=4) were transfected with scrambled or combined Twist1 or Twist2 siRNA. Cells were then pretreated with 0.5μg/ml IL-1Ra (blocks IL-1 consumption) and then treated with 100μg/ml MDP for 15 min. Supernatants were examined for IL-1β. **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$. Tx, treatment.

# Supplemental Figure 3




**Supplemental Figure 3. Multiple PRRs regulate expression of Twist1 and Twist2 and downstream signals.** **(A)** MDMs were left untreated (for acute) or pre-treated with 10μg/ml MDP, 0.1μg/ml Lipid A, 10μg/ml Pam3Cys or 1μg/ml CpG DNA alone or in combination for 48h, then treated with the same stimuli for an additional 24h (acute). Supernatants were examined for cytokines. Mean + SEM for n=4. **(B-D)** MDMs (n=8) were left untreated or pre-treated with 10μg/ml MDP, 0.1μg/ml Lipid A, 10μg/ml Pam3Cys or 1μg/ml CpG DNA alone or in combination for 48h, and then MDMs were treated with the same respective stimuli for an additional 4h (=chronic). Fold change in **(B)** Twist1, Twist2, **(C)** c-Maf, Bmi1, or **(D)** ATF4, C/EBPα, Runx1 or Runx2 mRNA normalized to untreated MDMs + SEM. Significance was assessed in treated relative to untreated cells, or as indicated. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times10^{-4}$; ††, $p<1\times10^{-5}$. Tx, treatment.

# Supplemental Figure 4




**Supplemental Figure 4. Schematic diagram of mechanisms for Twist1- and Twist2-mediated inhibition of cytokine secretion upon chronic NOD2 stimulation**. Acute NOD2 stimulation in primary human MDMs results in increased secretion of both pro-inflammatory and anti-inflammatory cytokines. With prolonged NOD2 stimulation, Twist1 and Twist2 expression increases in an autocrine IL-10- and TGFβ-dependent manner. The transcriptional repressors Twist1 and Twist2 bind to cytokine promoters and contribute to the downregulation of cytokines after acute NOD2 stimulation, and in particular, after chronic NOD2 stimulation. Twist1 and Twist2 also upregulate the expression and cytokine promoter binding of the anti-inflammatory transcription factors c-Maf and Bmi1, which in turn downregulates cytokine secretion. At the same time, Twist1 and Twist2 downregulate the expression and cytokine promoter binding of various activating transcription factors, including ATF4, C/EBPα, Runx1 and Runx2, which also contributes to the downregulation of cytokine expression upon NOD2 stimulation. Furthermore, chronic stimulation of additional PRRs, including TLR2, TLR4, and TLR9, can similarly upregulate Twist1 and Twist2 expression and downstream pathways. Moreover, these PRRs can cooperate with NOD2 to further enhance the regulatory outcomes. Therefore, Twist1 and Twist2 coordinate reciprocal regulation in expression and cytokine promoter binding of transcriptional repressors (increased) and transcriptional activators (decreased), which ultimately contributes to the cytokine downregulation observed after chronic PRR stimulation.