time course experiment monitoring RIG-I, IFN-β1, IL-6 and RANTES transcript induction within the first 8 hours after transfection of A549 cells with splicing reactions or fully purified circRNA did not reveal a transient response to circRNA (Figure 9M). Purified circRNA similarly failed to induce pro-inflammatory transcripts in RAW264.7 murine macrophages (Figure 9N). To generalize these findings to another synthetic circRNA construct, we tested the induction of pro-inflammatory transcripts in response to transfection of A549 cells with purified circRNA containing an EMCV IRES and EGFP coding region, and again failed to observe substantial induction (Figure 9O). These data demonstrate that non-circular components of the splicing reaction are responsible for the immunogenicity observed in previous studies and that circRNA is not a natural ligand for RIG-I.

[00214] Non-circular components of the splicing reaction contribute to immunogenicity

[00215] To explore the source of immunogenicity in circRNA splicing reactions, each component of the splicing reaction was purified by HPLC and assessed cytokine release and cell viability upon transfection of A549 cells (FIGS. 8A and B). Because there was difficulty obtaining suitably pure linear precursor RNA from the splicing reaction, precursor RNA in the form of the splice site deletion mutant (AS) (FIG. 8B, bottom right) was separately synthesized and purified. Additionally, the circRNA peak was split into two fractions to control for nicked RNA peak overlap (FIG. 8B). Robust IL-6, RANTES, and IP-10 release was observed in response to most species present within the splicing reaction as well as precursor RNA (FIG. 8C, FIG. 9G). Early circRNA fractions elicited cytokine responses comparable to other non-circRNA fractions, indicating that even relatively small quantities of linear RNA contaminants are able to induce a substantial cellular immune response in A549 cells. Late circRNA fractions elicited no cytokine response in excess of that from untransfected controls. Consistent with cytokine release observations, A549 cell viability 36 hours post transfection was significantly greater for late circRNA fractions compared to all other fractions (FIG. 8D).

[00216] Because it has been previously reported that circRNA may induce RIG-I transcription in a self-regulatory feedback loop, RIG-I and IFN-β1 transcript induction was analyzed upon transfection of A549 cells with late circRNA HPLC fractions (Chen, Y. G. et al. Sensing Self and Foreign Circular RNAs by Intron Identity. Mol. Cell 67, 228-238.e5 (2017)). A significantly weaker induction of both RIG-I and IFN-β1 transcripts for late circRNA fractions was observed