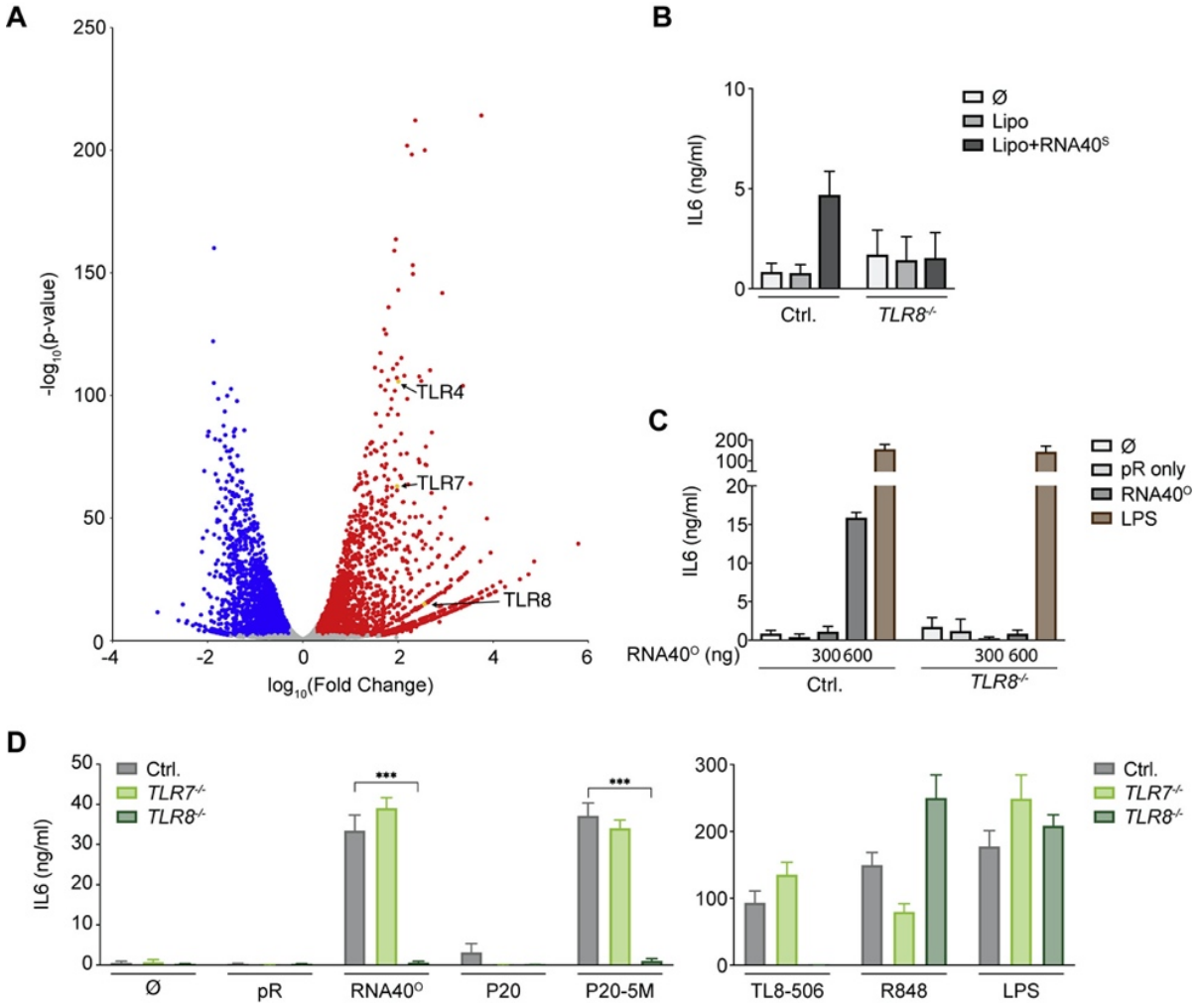

Figure S1. Characterization of TLR7 and TLR8 in BLaER1 Monocytes, Related to Figure 1

(A) Volcano plot showing gene expression differences of differentiated versus un-differentiated BLaER1 cells. The negative $\log_{10}$ pvalues (y axis) are plotted against the $\log_{10}$ fold changes in gene expression (x axis). Significantly (adjusted p-value <0.05 and absolute fold change >2) upregulated genes are highlighted in red, downregulated genes are highlighted in blue. TLR4, 7 and 8 are specifically highlighted. **(B)** BLaER1 Ctrl. and *TLR8*$^{-/-}$ cells were stimulated using Lipofectamine 2000 with and without RNA40^{S}. **(C)** BLaER1 cells were stimulated with different amounts of RNA40^{O}. The unstimulated control shown in (B) and (C) is the same as in Figure 1D, as it is derived from the same experiment. **(D)** Different RNAs were tested for TLR8 activation. P20 is a self-complementary RNA forming hairpins, whereas P20-5M is only a partially self-complementary RNA. Data are depicted as mean + SEM of three (B and C) or four (D) independent experiments. Statistics indicates significance by two-way ANOVA: $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, ns = not significant.

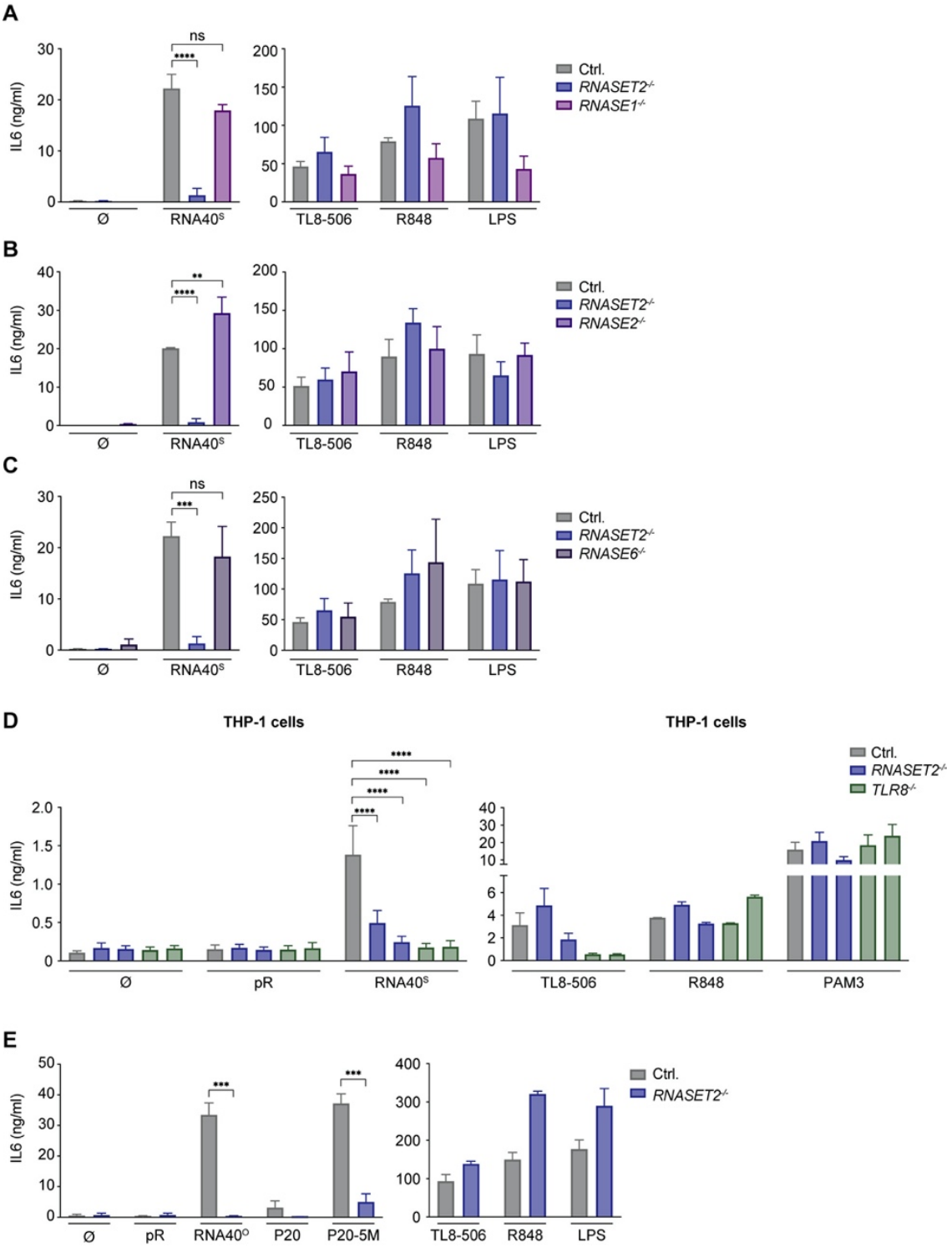

Figure S2. *RNASE1*$^{-/-}$, *RNASE2*$^{-/-}$, and *RNASE6*$^{-/-}$ Cells Show No Reduced Response to RNA40 Stimulation, Related to Figure 2

(A–C) BLaER1 control, *RNASE1, RNASE2* and *RNASE6*-deficient cells were stimulated with RNA40^{S}, TL8-506, R848 and LPS. Controls are identical for *RNASE1*$^{-/-}$ and *RNASE6*$^{-/-}$ cells as they derive from the same experiment. **(D)** THP-1 wild type, *RNASET2*$^{-/-}$ *and TLR8*$^{-/-}$ cells were stimulated as indicated. **(E)** BLaER1 control and *RNASET2*$^{-/-}$ cells were stimulated as indicated. Data of Ctrl. cells are identical to the ones in Figure S1D as they derive from the same experiment. Data are depicted as mean + SEM of three (A–D) or four (E) independent experiments. Statistics indicates significance by two-way ANOVA: $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, ns = not significant.

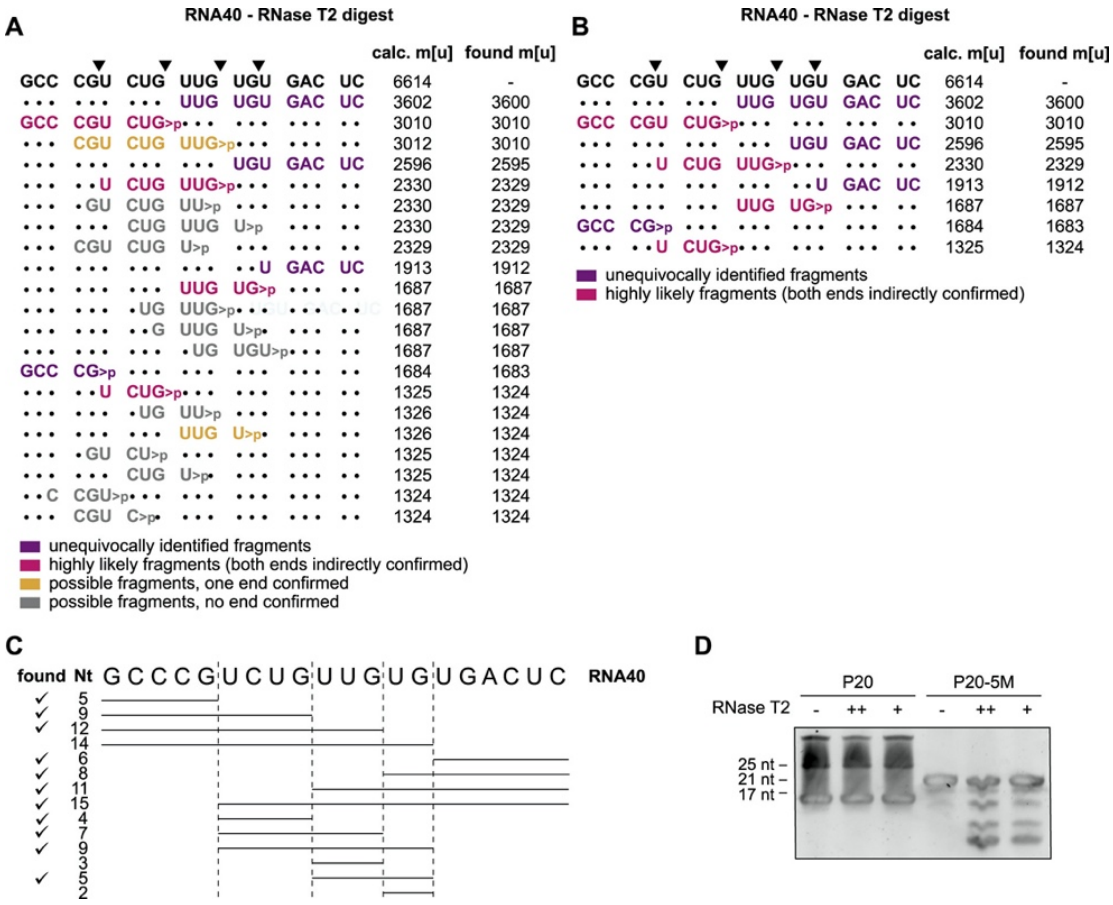

Figure S3. Identifying Possible RNase T2 Degradation Products by *In Vitro* Digestion of RNA40, Related to Figure 3

(A) List of all possible fragments associated with the *in vitro* digest of RNA40^S with RNase T2, analyzed by HPLC/MALDI-TOF. All found and calculated ($[M-H]^-$) masses are shown. The color code refers to the probability that fragments could be assigned to the one depicted. **(B)** Most likely hits of RNA40^S digested with RNase T2 based on the analysis from (A). **(C)** Assuming RNase T2 is only capable of cleaving RNA40 between G and U residues, all possible fragments are depicted. The ticks indicate which of these fragments could be confirmed by HPLC/MALDI-TOF. **(D)** Urea gel of *in vitro* digested RNA P20 and P20-5M with RNase T2. P20 is a self-complementary RNA fragment and forms hairpins, whereas P20-5M is only partially self-complementary RNA. One representative gel of two independent experiments is shown.

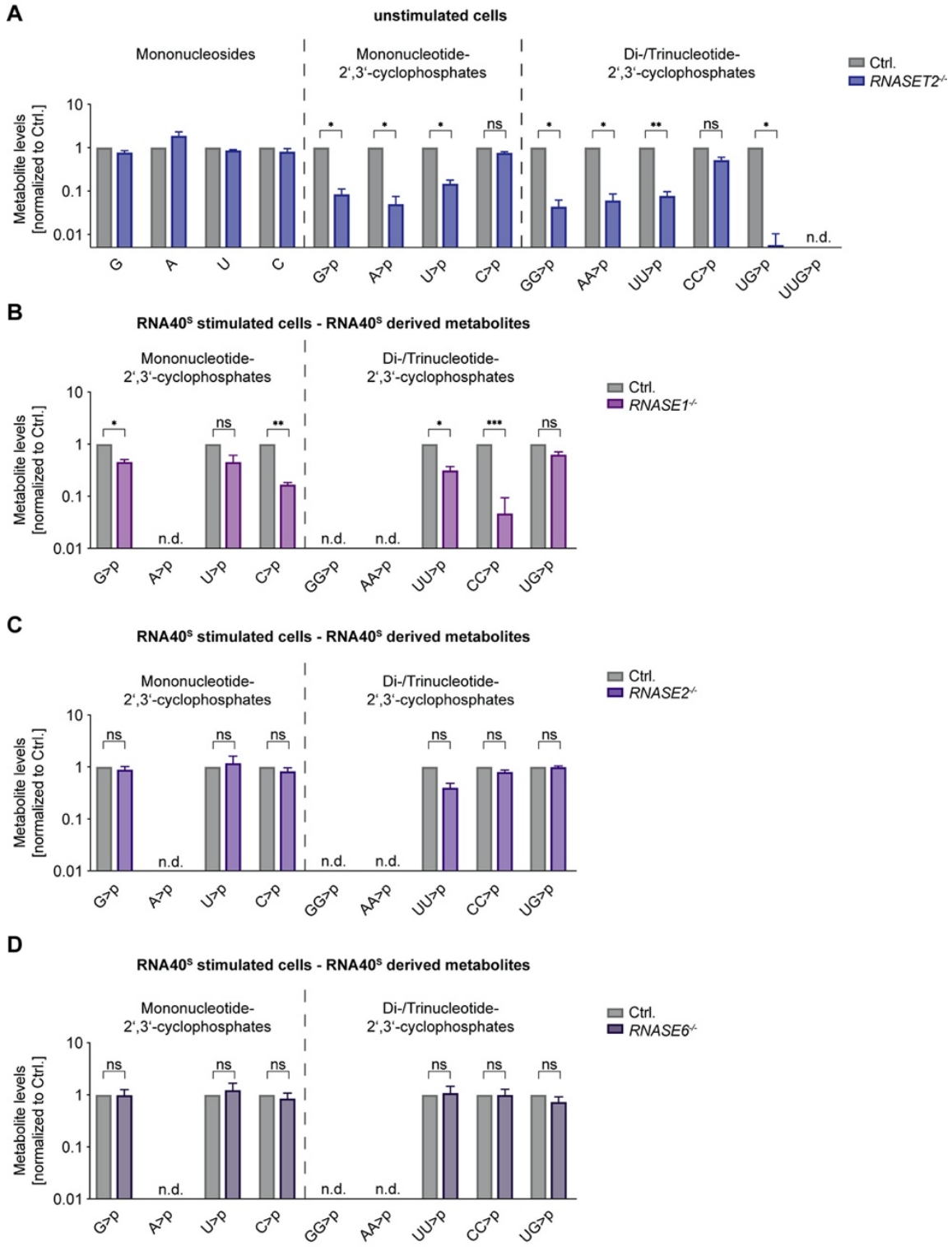

Figure S4. Cells Deficient in RNase A Family Enzymes Show Different RNA40 Catabolism in Comparison with *RNASET2*$^{-/-}$ Cells, Related to Figure 4

(A) Whole cell lysate of unstimulated BLaER1 cells with indicated genotypes were analyzed by LC-MS. Data are normalized to Ctrl. cells (note logarithmic scale). **(B–D)** Whole cell lysate of control, *RNASE1*$^{-/-}$, *RNASE2*$^{-/-}$ and *RNASE6*$^{-/-}$ BLaER1 cells stimulated with RNA40^{S} was analyzed by LC/MS (14 h after stimulation). RNA40^{S} derived metabolites are shown. Data are normalized to Ctrl. cells (note logarithmic scale). Data are depicted as mean + SEM of three independent experiments. Statistics indicates significance by a Welch's unequal variances t test. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$; ns, not significant; n.d., not detected.

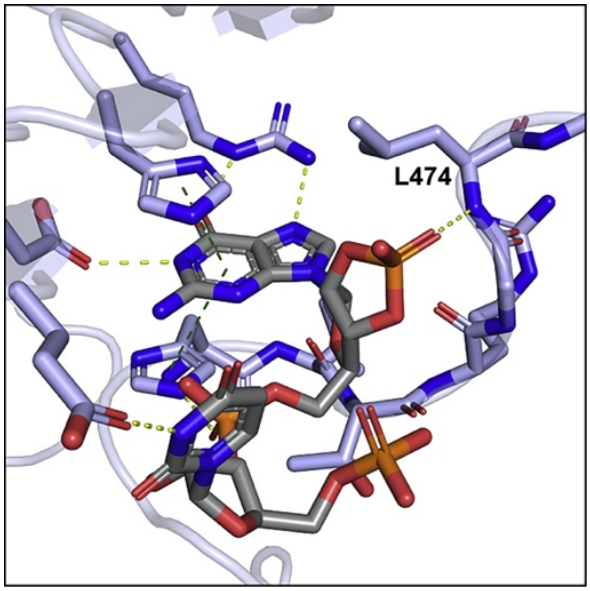

Figure S5. Crystal Structure of the Second Pocket of TLR8, Related to Figure 5

Crystal structure of the second pocket of TLR8 bound to UG (PDB, 4R07). Yellow dashed lines indicate hydrogen bonds whereas green dashed lines show π- π-interactions. The atoms are color coded as follows: nitrogen, blue; oxygen, red; phosphor, orange; carbon, light blue and gray.

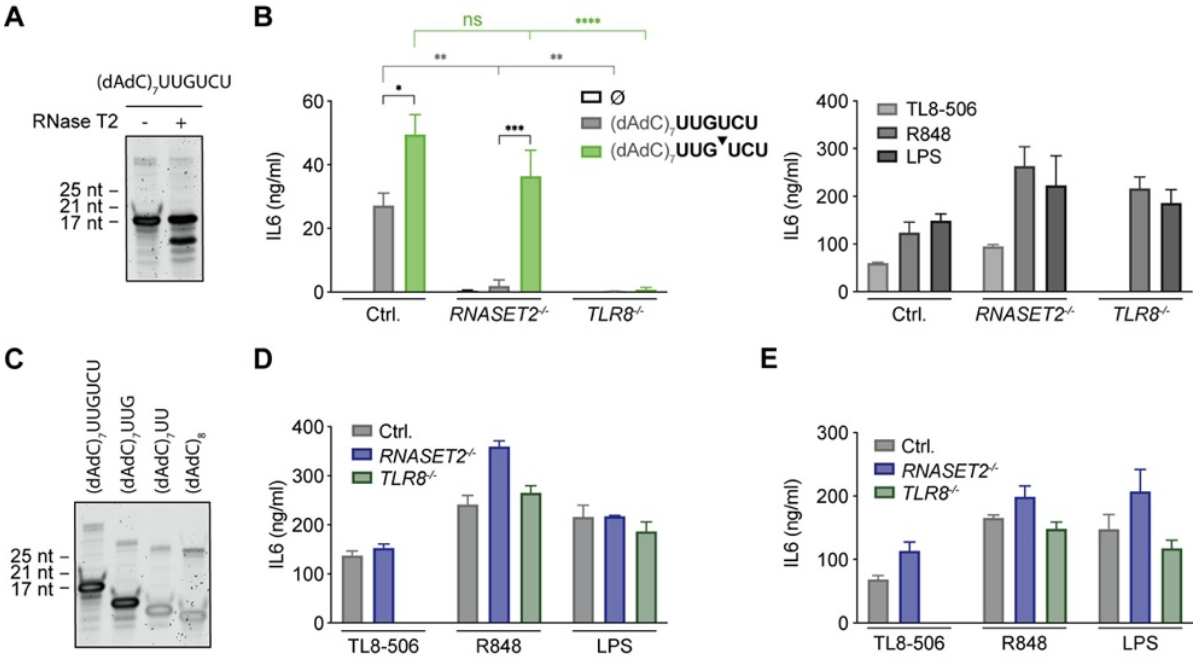

Figure S6. RNase T2 Degradation Products Bypass the Lack of RNase T2, Related to Figure 6

(A) Urea gel of full-length and RNase T2 digested $(dAdC)_7UUGUCU$. **(B)** BLaER1 cells of indicated genotypes were stimulated with either digested or undigested $(dAdC)_7$-UUGUCU (0.9 µg/condition) and the indicated controls. **(C)** Urea gel of the following full-length ONs: $(dAdC)_7$-UUGUCU, $(dAdC)_7$-UUG, $(dAdC)_7$-UU and $(dAdC)_8$. **(D)** Control stimulation of BLaER1 cells associated with (Figure 6G). **(E)** Control stimulation of BLaER1cells associated with (Figure 6H). Data are depicted as mean + SEM of three independent experiments or one of two representative gels is shown. Statistics indicates significance by two-way ANOVA. $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, ns = not significant.

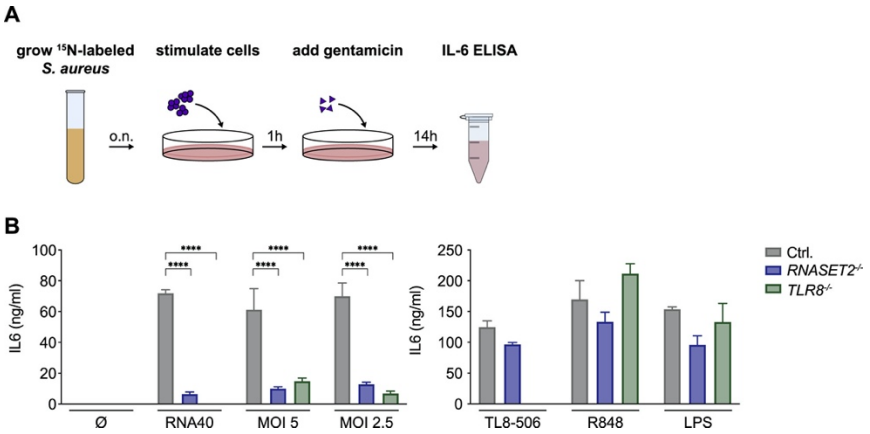

Figure S7. ^{15}N-Labeled *S. aureus* Detection in Myeloid Cells Depends on RNase T2 Upstream of TLR8, Related to Figure 7

(A) Schematic overview of experimental setup. *S. aureus* was grown in ^{15}N-labeled medium, harvested and used to stimulate differentiated BLaER1 cells. 1 h after infection gentamicin was added and another 14 h later IL-6 release was measured by ELISA. **(B)** Stimulation of BLaER1 cells with different MOI of ^{15}N-labeled *S. aureus*. Data are depicted as mean + SEM of three independent experiments. Statistics indicates significance by two-way ANOVA. $^{****}p \leq 0.0001$; $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, ns = not significant.