RTX-321, an Allogeneic Red Blood Cell–Based Artificial Antigen Presenting Cell, Expressing MHC I Peptide, 4-1BBL, and IL-12, Engages Primary Human HPV-Specific T Cells, and Boosts Other General Immune Responses

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Human papillomavirus (HPV) 16 is associated with approximately 70% of cervical cancers, approximately 40% of head and neck squamous cell carcinoma (HNSCC) arising in the oropharynx, approximately 25%-40% of HNSCC arising in other locations, and approximately 80%-85% of anal cancers.\textsuperscript{1-3}

Despite available therapies, a critical need remains for new treatment options for advanced HPV 16-associated cancers. To address this, we have genetically engineered cultured red blood cells to create an allogeneic artificial antigen presenting cell (aAPC), RTX-321, that expresses an HPV E7 peptide bound to major histocompatibility complex (MHC) I (Human leukocyte antigen [HLA]-A*02:01), 4-1BB ligand (4-1BBL) and interleukin-12 (IL-12) on the cell surface to mimic the biology of T cell-APC interactions. RTX-321 is designed to enhance both the quantity and quality of endogenous tumor-specific T cells.

The RED PLATFORM® is Designed to Generate Allogeneic, Off-the-Shelf Cellular Therapies

- **Red Cell Therapeutics™ (RCTs™)** are a new class of allogeneic, off-the-shelf cellular therapeutic candidates for the treatment of cancer and autoimmune diseases
- RCTs are enucleated reticulocytes that express hundreds of thousands of copies of biotherapeutic proteins on the cell surface
- Universal, scalable, and consistent manufacturing process

MHC = major histocompatibility complex.
Objectives

- To determine the ability of RTX-321 to induce activation and expansion of HPV 16 antigen-specific CD8+ T cells from a heterogeneous population of immune cells in peripheral blood mononuclear cells (PBMCs)
- To determine the ability of RTX-321 to induce HPV-independent adaptive and innate immune responses in vitro
RTX-321 is a Cellular Therapy with a Dual Mechanism of Action

RTX-321 consists of allogeneic, cultured, human-enucleated red blood cells engineered to express HPV 16 oncoprotein E7 peptide, presented on HLA-A*02:01 and β2 microglobulin (HLA-A2-HPV; Signal 1), 4-1BBL (tumor necrosis factor superfamily member 9; Signal 2), and a fusion protein of IL-12 (Signal 3) p40 and p35 subunits on the cell surface.

MHC = major histocompatibility complex; RTX-321 = RTX-HPV-4-1BBL-IL-12 product candidate; TCR = T cell receptor.
In order to investigate the dual mechanism of RTX-321 on innate and adaptive cells in vitro, we studied PBMCs alone and PBMCs plus engineered HPV 16 antigen-specific CD8+ T cells. This allowed us to analyze antigen-dependent and -independent immune responses.
Expression Profile of Cognate HPV E7 Antigen-Specific T Cells

RESULTS

E7 TCR expression was determined by HPV-specific tetramer. (A) PBMC alone conditions had no or minimal expression of HPV E7 TCR+ CD8 T cells (B) PBMC + E7 TCR-T cells expression ranged from 5.17-15.3% in two independent studies.

HPV = HLA-A2-human papillomavirus; PBMCs = peripheral blood mononuclear cells; TCR = T cell receptor.
RTX-321 Induces the Expansion of HPV 16 Antigen-Specific CD8+ T Cells

RESULTS

A. HPV-16 Antigen-Specific CD8+ T Cell Number

B. Total CD8+ T Cell Number

PBMCs alone, RCT-CTRL treated
PBMCs alone, RTX-321 treated
PBMC + E7 TCR-T cells, RCT-CTRL treated
PBMC + E7 TCR-T cells, RTX-321 treated

CD8+Tetramer+ Number

CD3+CD8+ T Cell Number
RTX-321 Induces the Expansion of HPV 16 Antigen-Specific CD8+ T Cells

SUMMARY
• RTX-321 selectively expands antigen-specific CD8+ T cells in the presence of other immune cells in vitro

STUDY DESIGN
RTX-321 or RCT-CTRL (8x10^5, 2x10^5, 5x10^4, or 1.25x10^4) were incubated with 2x10^5 PMBCs alone or 2x10^5 PBMC + 4x10^3 E7 TCR-T cells. To determine if RTX-321 can induce CD8+ T-cell expansion, the number of (A) HPV 16 antigen-specific CD8+ T cells and (B) total CD8+ T cells were measured by flow cytometry after 5 days of RTX-321 or RCT-CTRL treatment. Data are presented as mean ± standard deviation of duplicate wells from 4 donors. Each treatment was compared via 2-way ANOVA to the RCT-CTRL at the same dose and the same coincubation condition: *P<0.05, **P<0.001.

RCT-CTRL = untransduced control; HPV = HLA-A2-human papillomavirus; RTX-321 = RTX-HPV-4-1BBL-IL-12; TCR = T cell receptor.
RTX-321 Induces Activation, Proliferation, Effector Upregulation, and Increases $T_{\text{EM}}$ Phenotype in HPV E7 Antigen-Specific CD8+ T cells

**RESULTS**

**A.** CD25+%

**B.** Ki67+%

**C.** GZMB+%

**D.** T-bet+%

**E.** $T_{\text{EM}}$+%

[Graphs showing percentage of live total CD8 cells for each category with statistical significance indicated by asterisks.]
STUDY DESIGN

RTX-321 or RCT-CTRL (8x10^5, 2x10^5, 5x10^4, or 1.25x10^4) were incubated with 2x10^5 PMBCs alone or 2x10^5 PBMC + 4x10^3 E7 TCR-T cells. To determine if RTX-321 can induce activation, proliferation, effector upregulation and increase T<sub>EM</sub>+ phenotype in CD8+ T cells, the percentage of (A) CD25+; (B) Ki67+; (C) GZM<sub>B</sub>+; (D) T-bet+; and (E) T<sub>EM</sub>+ in CD8+ T cells were measured by flow cytometry after 5 days of RTX-321 or RCT-CTRL treatment. Data are presented as mean ± standard deviation of duplicate wells from 3 or 4 donors. Each treatment was compared via 2-way ANOVA to the RCT-CTRL at the same dose and the same coincubation condition: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

RCT-CTRL = untransduced control; HPV = HLA-A2-human papillomavirus; RTX-321 = RTX-HPV-4-1BBL-IL-12; TCR = T cell receptor; GZMB = Granzyme B; CD25 = Cluster of Differentiation 25; T-bet = T-box expressed in T cells; T<sub>EM</sub> = effector memory T cell.

SUMMARY

• RTX-321 induces activation (CD25), proliferation (Ki67), effector function/potential (GZMB and T-bet) and increases T<sub>EM</sub> phenotype on CD8+ T cells in an antigen dependent manner
RTX-321 Induces Activation, Proliferation, Effector Upregulation, and Increases T_{EM} Phenotype in HPV 16 and Non-HPV Antigen-Specific CD8+ T Cells

RESULTS

A. CD25+%

B. HLA-DR+%

C. Ki67+%

D. GZMB%

E. T-bet+%

F. T_{EM}+%

Tetramer+ CD8, treated with RTX-321

Tetramer- CD8, treated with RTX-321

Tetramer- CD8, treated with RTX-CTRL
RTX-321 Induces Activation, Proliferation, Effector Upregulation, and Increases $T_{EM}$ Phenotype in HPV 16 and Non-HPV Antigen-Specific CD8+ T Cells

**STUDY DESIGN**

RTX-321 or RCT-CTRL (8x10^5, 2x10^5, 5x10^4 or 1.25x10^4) were incubated with 2x10^5 PMBCs alone or 2x10^5 PBMC + 4x10^3 E7 TCR-T cells. To compare RTX-321 induced activation, proliferation, effector upregulation and increased $T_{EM}$ phenotype in HPV 16 antigen-specific CD8+ T cells (tetramer+) and non-HPV 16 antigen-specific CD8+ T cells (tetramer-) in PBMC + E7 TCR-T cells, the percentage of (A) CD25+; (B) HLA-DR+; (C) Ki67+; (D) GZMB+; (E) T-bet+; and (F) $T_{EM}$ in tetramer+ or tetramer- cells were measured by flow cytometry after 5 days of RTX-321 or RCT-CTRL treatment. Data are presented as mean ± standard deviation of duplicate wells from 3 or 4 donors. 2-way ANOVA with Tukey’s multiple comparison for overall treatment effects: *P<0.05, **P<0.01, ****P<0.0001.

RCT-CTRL = untransduced control; HPV = HLA-A2-human papillomavirus; RTX-321 = RTX-HPV-4-1BBL-IL-12; TCR = T cell receptor; GZMB = Granzyme B; CD25 = Cluster of Differentiation 25; HLA-DR = Human Leukocyte Antigen – DR; T-bet = T-box expressed in T cells ; $T_{EM}$ = effector memory T cell.

**SUMMARY**

- RTX-321 induces activation, proliferation, effector function/potential and increases $T_{EM}$ phenotype on HPV 16 antigen-specific CD8+ T cells and non-HPV 16 antigen-specific CD8+ T cells
RTX-321 Induces the Expansion of NK Cells and NK Subsets

**RESULTS**

A. NK number

B. CD16+ NK number

C. CD16- NK number

- PBMCs alone, RCT-CTRL treated
- PBMCs alone, RTX-321 treated
- PBMC + E7 TCR-T cells, RCT-CTRL treated
- PBMC + E7 TCR-T cells, RTX-321 treated
RTX-321 Induces the Expansion of NK Cells and NK Subsets

STUDY DESIGN

RTX-321 or RCT-CTRL (1.25x10^4, 5x10^4, 2x10^5 or 8x10^5) were incubated with 2x10^5 PMBCs alone or 2x10^5 PBMC + 4x10^3 E7 TCR-T cells. To determine the effects of RTX-321 on NK cell expansion, total NK cell numbers, and numbers of CD16+ and CD16- NK subsets were determined by flow cytometry 9 days after RTX-321 or RCT-CTRL treatment. Data are presented as mean ± standard deviation of duplicate wells from 3 or 4 donors. Each treatment was compared via 2-way ANOVA to the RCT-CTRL at the same dose and the same coincubation condition: **P<0.01, ***P<0.001, ****P<0.0001.

RCT-CTRL = untransduced control; HPV = HLA-A2-human papillomavirus; RTX-321 = RTX-HPV-4-1BBL-IL-12; CD16 = FcγRIII.

SUMMARY

- RTX-321 expands total NK cells and NK subsets (CD16+ NK and CD16- NK) independent of the presence of HPV 16 antigen-specific CD8+ T cells
RTX-321 Induces the Activation, Proliferation and Effector Upregulation of NK Cells

RESULTS

A. NKp30+

B. 4-1BB+

C. DNAM-1+

D. CD25+

E. Ki67+

F. GZMB+

G. T-bet+

PBMCs alone, RCT-CTRL treated
PBMCs alone, RTX-321 treated
PBMC + E7 TCR-T cells, RCT-CTRL treated
PBMC + E7 TCR-T cells, RTX-321 treated
RTX-321 Induces the Activation, Proliferation and Effector Upregulation of NK Cells

**STUDY DESIGN**

RTX-321 or RCT-CTRL (8x10^5, 2x10^5, 5x10^4, or 1.25x10^4) were incubated with 2x10^5 PMBCs alone or 2x10^5 PBMCs + 4x10^3 E7 TCR-T cells. To determine the effects of RTX-321 on NK cell activation, proliferation and effector upregulation, the percentage of (A) NKp30+ on day 5; (B) 4-1BB+ on day 8; (C) DNAM1+ on day 8; (D) CD25+ on day 9; (E) Ki67+ on day 5; (F) GZMB+ on day 5; and (G) T-bet+ on day 8 on total NK cells were measured by flow cytometry 5, 8, or 9 days after RTX-321 or RCT-CTRL treatments. Data are presented as mean ± standard deviation of duplicate wells from 3 or 4 donors. Each treatment was compared via 2-way ANOVA to the RCT-CTRL at the same dose and the same coincubation condition: *P<0.05 ** P<0.01, ***P<0.001, ****P<0.0001.

RCT-CTRL = untransduced control; HPV = HLA-A2-human papillomavirus; RTX-321 = RTX-HPV-4-1BBL-IL-12; NKp30 = Natural killer cell p30-related protein; CD25 = Cluster of Differentiation 25, 4-1BB = CD137, TNFRS9; DNAM-1 = DNAX accessory molecule 1; HLA-DR = Human Leukocyte Antigen – DR; GZMB = Granzyme B; T-bet = T-box expressed in T cells; T_EM = effector memory T cell.

**SUMMARY**

- RTX-321 increased activation (NKp30, 4-1BB, DNAM-1, and CD25), proliferation (Ki67) and effector function/potential (GZMB and T-bet) on total NK cells, independent of the presence of HPV 16 antigen-specific CD8+ T cells
RTX-321 Induces Effector Molecule Secretion and 4-1BB Shedding in Total PBMCs

RESULTS

PBMCs alone, RCT CTRL treated
PBMCs alone, RTX-321 treated
PBMC + E7 TCR-T cells, RCT CTRL treated
PBMC + E7 TCR-T cells, RTX-321 treated

A. IFNγ

B. TNFα

C. CXCL10

D. Soluble 4-1BB

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RTX-321 Induces Effector Molecule Secretion and 4-1BB Shedding in Total PBMCs

**STUDY DESIGN**

RTX-321 or RCT-CTRL (1.25 x10^4, 5x10^4, 2x10^5, or 8x10^5) were incubated with 2x10^5 PMBCs alone or 2x10^5 PBMC + 4x10^3 E7 TCR-T cells. To assess RTX-321 effects on cytokine and chemokine responses (A) IFNγ; (B) TNFα; (C) CXCL10 concentrations were quantified by MSD assay 9 days after RTX-321 or RCT-CTRL treatments; to determine RTX-321 effects on 4-1BB shedding, (D) soluble 4-1BB concentration was quantified by ELISA 9 days after RTX-321 or RCT-CTRL treatments. Data are presented as mean ± standard deviation of duplicate wells from 4 donors. Each treatment was compared via 2-way ANOVA to the RCT-CTRL at the same dose and the same coincubation condition: **P<0.01, ***P<0.001, ****P<0.0001.

RCT-CTRL = transduced control; HPV = HLA-A2-human papillomavirus; RCT= red cell therapeutic experimental construct; RTX-321 = RTX-HPV-4-1BBL-IL-12; IFNγ = Interferon gamma; TNFα = tumor necrosis factor alpha ; CXCL10 = C–X–C motif chemokine 10 ; soluble 4-1BB = Soluble CD137.

**SUMMARY**

- RTX-321 increased the secretion of IFNγ in PBMCs alone, which was further increased in the presence of HPV 16 antigen-specific CD8+ T cells
- RTX-321 increased the secretion of TNFα in the presence of HPV 16 antigen-specific CD8+ T cells and CXCL10 in PBMCs alone
- RTX-321 increased the 4-1BB shedding in PBMCs alone, which was further increased in the presence of HPV 16 antigen-specific CD8+ T cells indicating that RTX-321 can robustly engage its target receptor
Conclusions

• RTX-321 has a dual mechanism of action
  1) Functions as an aAPC to boost HPV 16 E7-specific CD8+ T-cell responses
  2) Promotes HPV 16-independent stimulation of innate (NK cells) and adaptive immune (non-HPV antigen-specific CD8+ T cells) responses

• These findings support the dual mechanism of action of RTX-321 and its potential as an effective therapy for cancers associated with HPV 16

• Rubius plans to file an Investigational New Drug application by the end of 2020
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DISCLOSURES
All authors: Employment with and equity ownership in Rubius Therapeutics.