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Killing tumor-associated bacteria with a liposomal antibiotic generates neoantigens that induce anti-tumor immune responses

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Indroduction

- tumor microbiota can infuence cancer progression
- design an antibiotic silver-tinidazole complex encapsulated in liposomes (LipoAgTNZ)
- mouse models with colorectal cancer responded to LipoAgTNZ therapy, allowing more than 70% survival for a long period of time

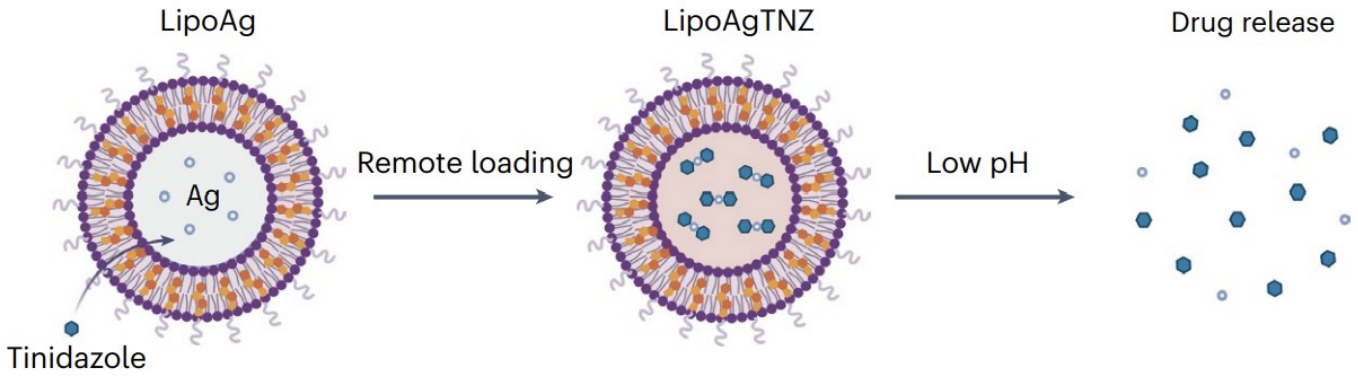

Illustration of remote loading by a silver nitrate gradient and drug release in response to low pH

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- The release of T-cell immunity to elicit anti-tumor immune responses has led to important clinical advances in the fight against cancer, including checkpoint inhibitors, cancer vaccines, and chimeric antigen receptor T-cell therapies

- **Elimination of the intracellular bacteria in the tumor will reveal microbial epitopes and produce neoantigens linked to cancer from different sources.**

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RESULTS

Antibiotics targeting anaerobes improved cancer survival

- selective targeting of oncogenic bacteria associated with cancer
- nitroimidazol and lincomycin
- database – resected patients with these antibiotic classes vs receiving other antibiotics vs no antibiotics
- improved disease-free survival (DFS)??
- 2012-2014 36 105 patients with colorectal cancer (CRC)
- 4 413 received antibiotic treatment 6 months before/12 months after resection
- hazard ratio (HR) was lower for antibiotics targeting anaerobes than without antibiotics among patients with CRC
- before resection of the tumor – protective effect
- tumor is a target lesion – reduce risk of recurrence or death by 25.5%

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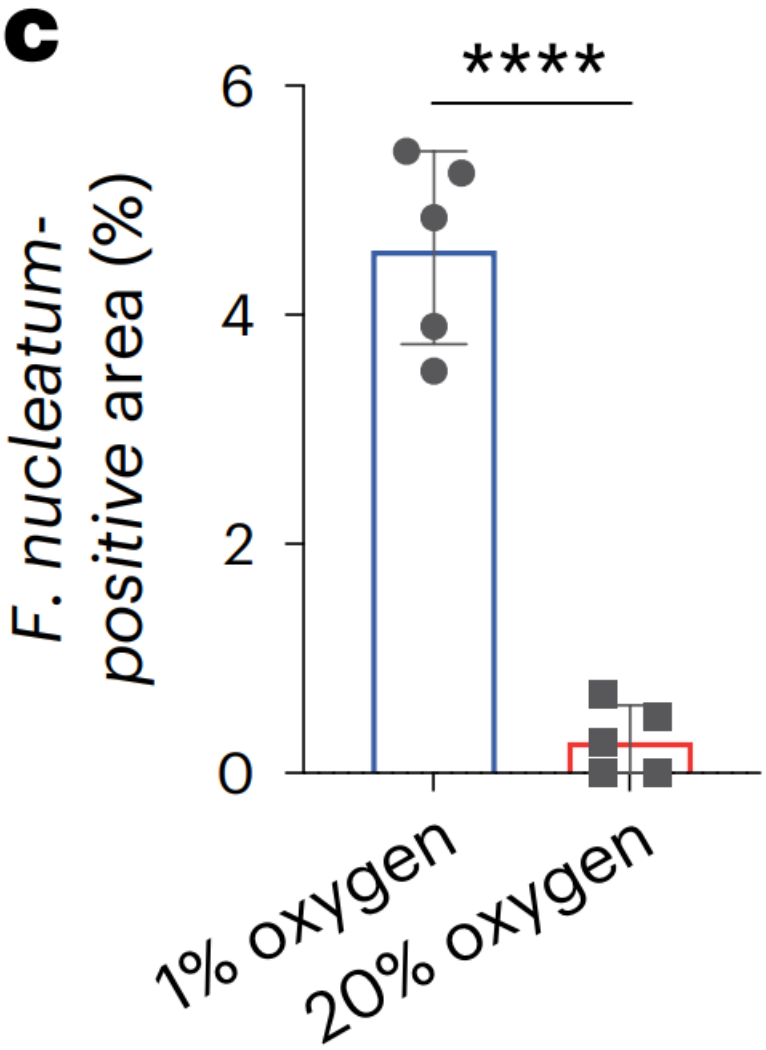
Antibiotics targeting anaerobes improved cancer survival

- specific role of tumor primary -> patients with breast cancer -> protective effect not found
- -> specific to CRC and its microbiota
- antibiotics targeting anaerobes vs other antibiotics ->DFS improved (no effect in breast cancer)
- hypothesis – specific antibiotics have the potential to reduce the risk of recurrence of CRC with an effect that may be as important as chemotherapy, which usually reduces the risk of recurrence by 32% or death by 26% after resection in combination with adjuvant treatments

Bacteria invaded tumor cells in response to low oxygen level

- gram-negative and anaerobic bacteria Fusobacterium nucleatum
- tumor hypoxia – 1% or 20% oxygen for 24h
- F. nucleatum was able to invade hypoxic tumor cells with higher signals
- F. nucleatum was able to invade inside the cytoskeleton in response to hypoxia
- **F. nucleatum has a stronger correlation with larger tumors with hypoxic regions**
- **Spontaneous invasion to hypoxic tumor cells was also found in the facultative anaerobic probiotic strain Escherichia coli Nissle -> bacteria preferentially invade hypoxic tumors**



Quantification of the F. nucleatum-positive area by CFSE fluorescence. n = 5 experiments. Data are the mean ± s.d. ****P < 0.0001.

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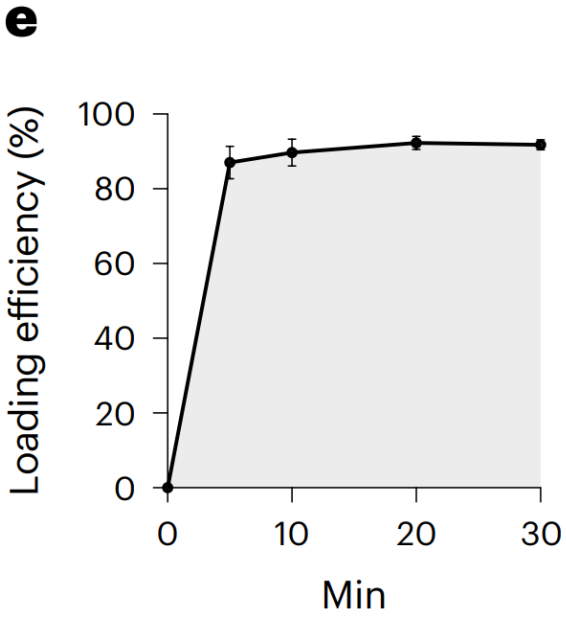
Bacteria invaded tumor cells in response to low oxygen level

- CRC in Balb/C mice infected with F. nucleasum - **higher tumor growth ratio compared to uninfected controls**
- F. nucleatum infection considerably **promoted tumor metastasis in proximal lymph nodes and distal metastasis**
- transmission electron microscopy (TEM) images of F. nucleatum infected CRC tumors – **intracellular in vivo in the tumor region**
- fluorescence in situ hybridization (FISH) with RNA probe specific for the 16S ribosomal RNA (rRNA) – **F. nucleatum near the cell nuclei in the CRC tumor**

Liposomal antibiotics eliminated Bacteria in the tumor

- Ag^+ - antibacterial agents used in various forms as antibiotics for centruries interaction with sufur and phosphorus – enzymes and DNA
- silver nanoparticles – ability to adhere and penetrate the bacteria cell wall
- cancer cells metabolized pyruvate into lactate and ethanol
- ↓ pH of hypoxic tumors – elevated levels of lactic acid – drug release in anaerobic bacteria residing in the region of the tumor
- metal ions can by trapping agents for loading nitroimidazol into liposomes
- loading of TNZ into silver-containing liposomes was efficient – more than 80% encapsulation efficiency

- liposomes – localized in tumor and liver – DiD fluorescence
- mice were injected with free drugs and liposomes – tinidazol encapsulated in liposomes mainly accumulated in the tumor 24h after injection – succes of tumor targetting
- toxicity biomarkers were assessed – no obvious alternations in serum biomarkers were observed compared to the untreated mice



Loading kinetics of LipoAgTNZ. n = 3 experiments.

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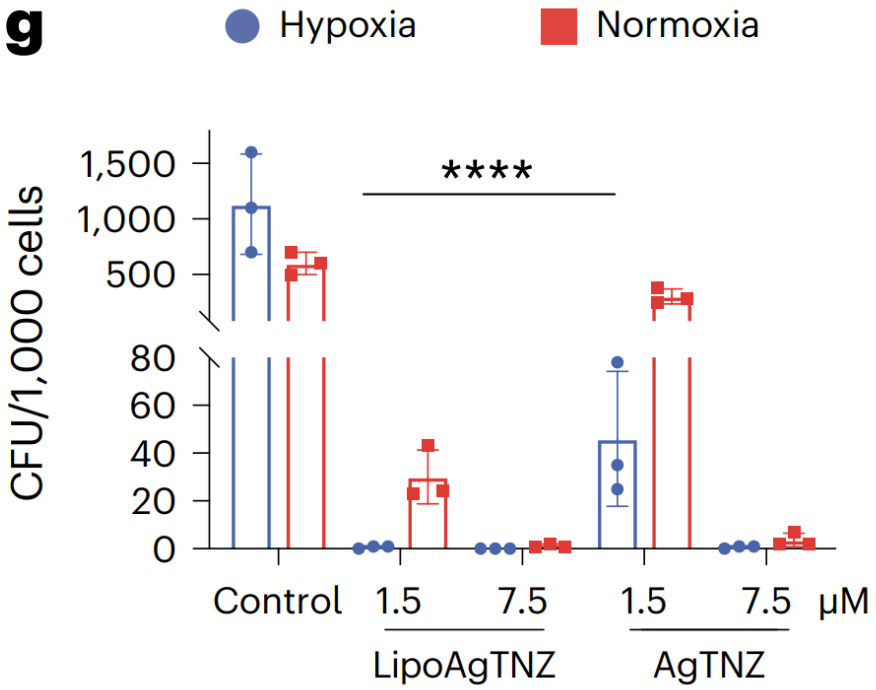

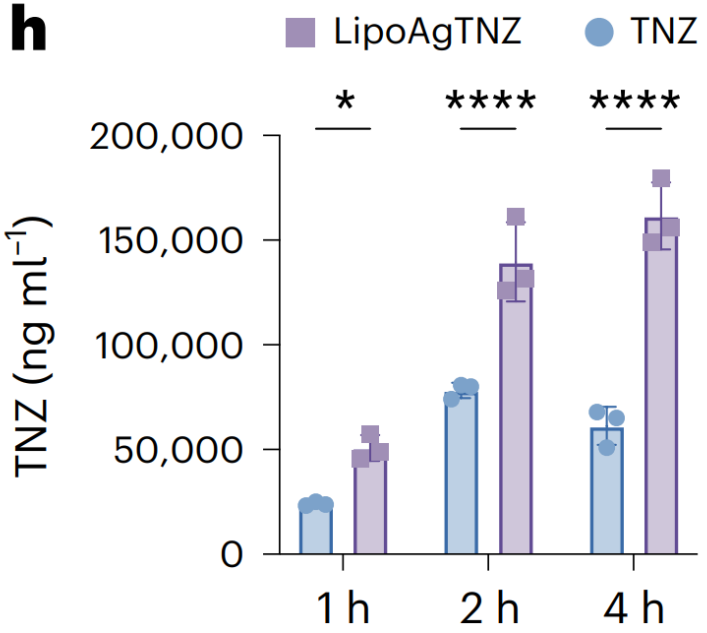

Intracellular F. nucleatum killing by free AgTNZ and LipoAgTNZ under normoxia and hypoxia. n = 3 experiments. Data are the mean ± s.d. ****P < 0.0001

- Both LipoAgTNZ and free AgTNZ showed higher antimicrobial efficacy in hypoxia.

In vitro cell uptake of liposomes and free drug at 1 h, 2 h and 4 h. n = 3 experiments. Data are the mean ± s.d. *P < 0.05; ****P < 0.0001.

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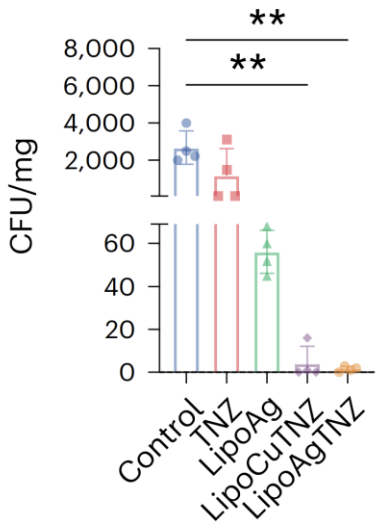
Killing intracellular bacteria improved immune surveillance

- CRC mouse model was used to test the therapeutic efficacy of the antibiotic liposomes (infected with F. nucleatum or E. coli Nissle)
- **The primary organs of the mice given LipoAgTNZ treatment did not show any signs of bacterial colonization**



CFU of F. nucleatum in the tumors at day 24

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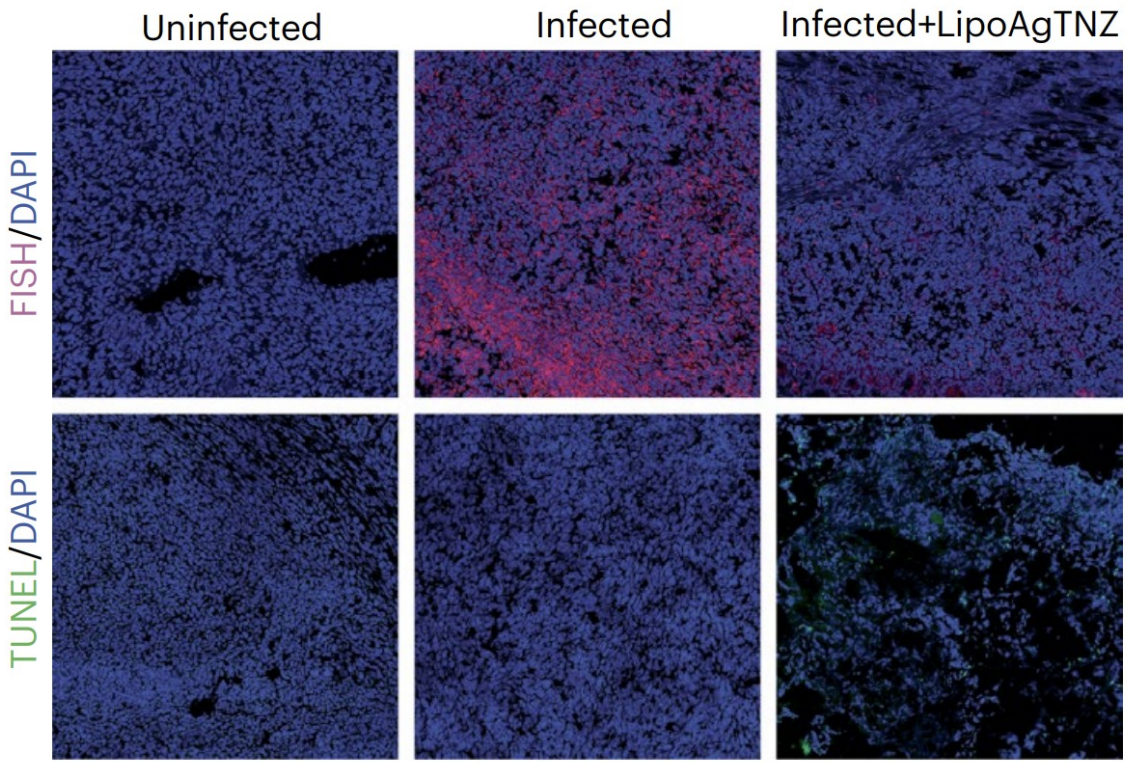

FISH assays visualizing F. nucleatum 16S RNA; TUNEL staining showing apoptosis

- **FISH** signals revealed that LipoAgTNZ effectively reduced the F. nucleatum burden in CRC tumors.
- **TUNEL** assay was performed and showed increased apoptotic cells after treatment

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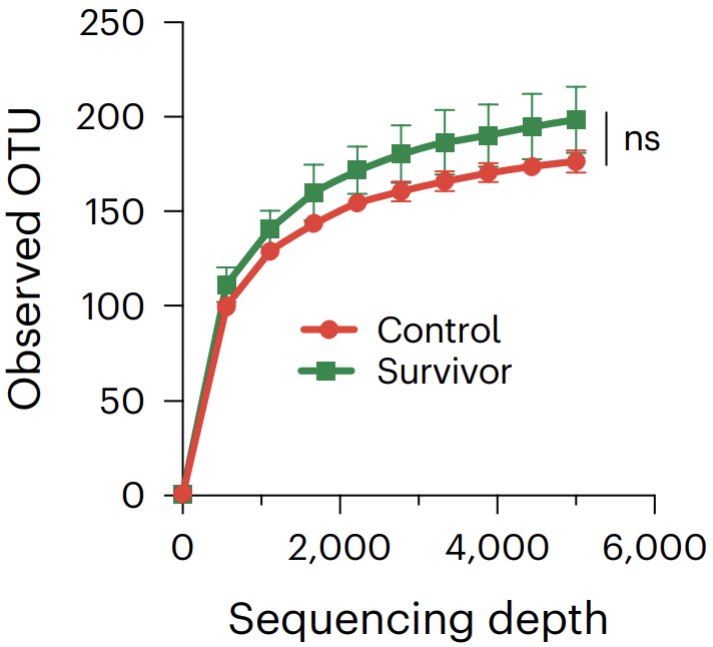

Alpha diversity measuring the microbiome diversity of each sample

- **Determination of gut microbiota**: high-throughput gene sequencing analysis of 16S rRNA in fecal bacterial DNA
- bacterial communities of survivors and controls were similar
- **Idea**: specific gut microbiota homeostasis was protected by using LipoAgTNZ treatment

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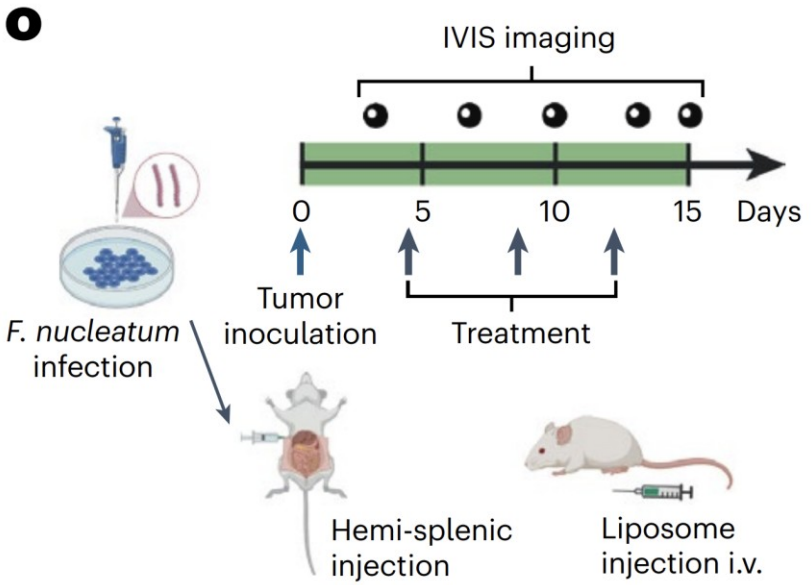

- **Liver metastasis**: most common distant metastasis in colorectal cancer,70% of patients
- **LipoAgTNZ liposomes**: effective destruction of bacteria in liver metastases and production of anti-tumor effects

Scheme of F. nucleatum-infected liver metastasis model.

The CT26FL3(Luc/RFP) cells were pre-infected with F. nucleatum and inoculated into the liver by hemi-splenic injection, followed by three doses of LipoAgTNZ treatment

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T cells from long-term survivors showed specificity to both infected and unifected tumors

- Comparison of T cells from survivor mice to age-matched, naive, uninfected mice - to study T cell specificity to bacterial infection
- The data again suggest that survivor T cells **recognized both** infected and uninfected tumor cells in vivo in the recipient mice

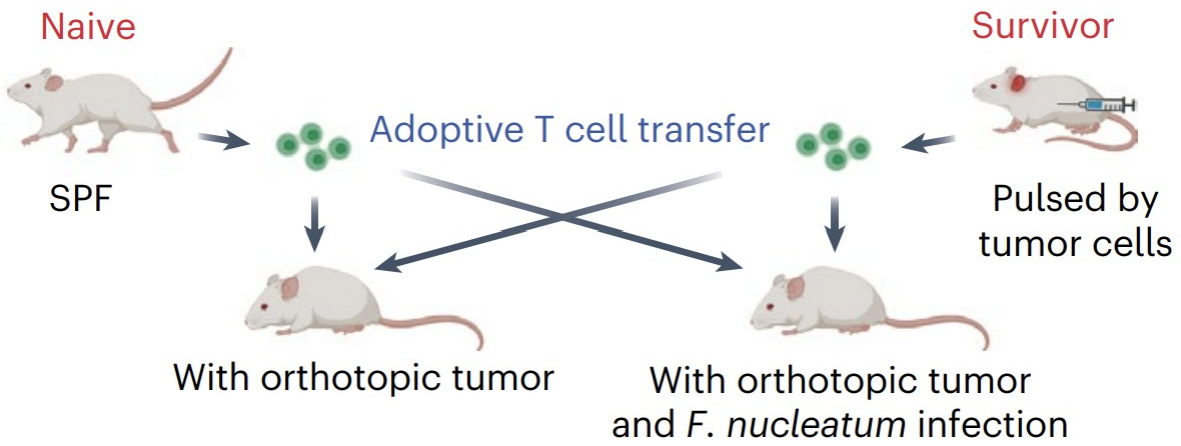

Illustration of the T cell adoptive transfer study.
Mice with orthotopic CRC tumors with or without F. nucleatum infection received T cells from either naive mice or long-term survivor mice from LipoAgTNZ treatment.

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Host T cells specifically targeted bacterial epitopes after antibiotic treatment

- Prediction of binding peptides (MHC-I H-2Kd, H-2Dd, and H2Ld) to estimated the potential neoepitopes
- selection of the epitopes - the most abundant cytoplasmic proteins were chosen
- peptides 1 through 5 - neoantigens derived from bacteria that do not share any sequence similarities with mice
- the homologous epitopes peptides 6–8 are shared by mice and bacteria

Protein	Epitope	Number	Peptide length	Allele	Amino acid matching	Similarity with host	Host protein with similarity
Neutrophil-activating protein A	KYLSNLGIL	1	9	H-2-Kd	9–17	N	N/A
Tryptophanase	LPMRHFHAF	2	9	H-2-Ld	48–56	N	N/A
NAD-specific glutamate dehydrogenase	SYFEWVQNI	3	9	H-2-Kd	368–376	N	N/A
Methionine gamma-lyase	MPIYQTSTF	4	9	H-2-Ld	27–35	N	N/A
3-hydroxybutyryl-CoA dehydrogenase	RPMIGMHFF	5	9	H-2-Ld	9–17	N	N/A
Chaperone protein DnaK	GVPQIEVTF	6	9	H-2-Dd	439–447	Y	Stress-70 protein
Chaperone protein DnaK	RQATKDAGTI	7	10	H-2-Kd	127–136	Y	Endoplasmic reticulum chaperone bip precursor
P-type Ca(2+) transporter	LADDNFSTIV	8	10	H-2-Kd	641–649	Y	Sarcoplasmic/Endoplasmic reticulum calcium atpase 2

Information on the selected peptides predicted by the Immune Epitope Database (IEDB) database

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- T cells from survivors established long-term anti-tumor efficacy compared to T cells from naive mice
- While **CD4+ T cells** in response to class II MHC antigens have been shown to produce either cytotoxicity or tolerance, they primarily examined bacteria-induced **CD8+ T cells** in response to class I MHC antigens which is the cytotoxic T cell phenotype for anti-tumor efficacy

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Conclusion

- **Killing of tumor-associated bacteria may contribute a new source of neoantigens to facilitate homologous and neoepitope-mediated cellular immunity against CRC**
- Antibiotics targeting anaerobic bacteria extended the DFS of patients with CRC and infected mice
- CRC patients – the benefit derived from antibiotics was limited to pre-resection intake
- Quantity of intratumoral bacteria to prime immunity may be crucial to succes
- Anti-tumoral immune response relies on the ability of immune cells to recognize and eliminate tumor-derived antigens
- Mutation-derived neoantigens are recognized as a major determinant to benefit from immunomodulating strategies

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Conclusion

- The developed liposomes remotely loaded with antibiotics to target the CR tumors
- They performer adoptive T cell transfer and T cell epitope studies to investigate the function of the immune system after bacterial removal
- Homologous epitopes also showed anti-tumor efficacy, which may result in an immune response to uninfected tumors
- Killing of tumor-associated Bacteria can turn an immunologically cold tumor into a hot tumor and prime the immune system to recognize both infected and uninfected tumor cells

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For the last time ☺

- The researchers used a liposomal antibiotic to target Fusobacterium nucleatum, a bacterium that has been associated with colorectal cancer, and tested the effects of this treatment on both bacterial load and immune response in vitro and in vivo. The study also aimed to identify potential biomarkers of response to the treatment and to explore the mechanisms underlying the observed effects.

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Thank you for your attention