Conjugating Prussian blue nanoparticles onto antigen-specific T cells as a combined nanoimmunotherapy

Aim: To engineer a novel nanoimmunotherapy comprising Prussian blue nanoparticles (PBNPs) conjugated to antigen-specific cytotoxic T lymphocytes (CTL), which leverages PBNPs for their photothermal therapy (PTT) capabilities and Epstein–Barr virus (EBV) antigen-specific CTL for their ability to traffic to and destroy EBV antigen-expressing target cells. **Materials & methods:** PBNPs and CTL were independently biofunctionalized. Subsequently, PBNPs were conjugated onto CTL using avidin–biotin interactions. The resultant cell-nanoparticle construct (CTL:PBNPs) were analyzed for their physical, phenotypic and functional properties. **Results:** Both PBNPs and CTL maintained their intrinsic physical, phenotypic and functional properties within the CTL:PBNPs. **Conclusion:** This study highlights the potential of our CTL:PBNPs nanoimmunotherapy as a novel therapeutic for treating virus-associated malignancies such as EBV+ cancers.

First submitted: 14 April 2016; Accepted for publication: 3 June 2016; Published online: 7 July 2016

Keywords: antigen-specific T cells • cell nanoparticle construct • cytotoxic T lymphocyte • immunotherapy • nanoimmunotherapy • photothermal therapy • Prussian blue nanoparticles

Nanoparticles have many promising therapeutic and imaging applications in medicine [1,2]. An emerging field in nanomedicine is the development of ‘nanoimmunotherapies’ that combine the advantages of nanoparticles with immune-based therapies, which harness the immune system for therapeutic use [3,4]. This combination nanoimmunotherapy approach offers the potential for the development of a robust and persistent therapeutic which can be applied to diverse disease settings such as infectious diseases and cancer [5–7].

In this communication, we describe a novel nanoimmunotherapy, which combines Prussian blue nanoparticles (PBNPs) with antigen-specific cytotoxic T lymphocytes (CTL). To develop our nanoimmunotherapy construct, we utilized a bioconjugation technique whereby nanoparticles are stably conjugated onto the surface of cells. Integrating nanoparticles with cells in a cell-

nanoparticle biohybrid has the potential to harness the intrinsic properties of both the nanoparticles (e.g., imaging, therapy) and the CTL (e.g., antitumor and/or antiviral responses) [8,9]. It is therefore critical that both the nanoparticles and the CTL retain their native properties during the attachment process.

Cell-nanoparticle constructs have been previously described for various applications *in vitro* and *in vivo*, including cell tracking applications and chemotherapy drug conjugation with monocytes and lymphocytes [8–11]. However, to our knowledge, none have used these unique PBNPs in combination with Epstein–Barr virus (EBV) antigen-specific T cells. As a nanoparticle, PBNPs offer several advantages: they are easily synthesized in a single, scalable step at low costs. We have previously demonstrated the successful use of PBNPs for PTT of tumors *in vivo* [12]

Rachel A Burga^{1,4,6},
Shabnum Patel^{1,6},
Catherine M Bollard^{1,2,5,6},
Conrad Russell Y Cruz^{*,1,2,5,6}
& Rohan Fernandes^{**,1,2,3,4}

¹Institute for Biomedical Sciences, The George Washington University, Washington, DC, USA

²Department of Pediatrics, The George Washington University, Washington, DC, USA

³Department of Radiology, The George Washington University, Washington, DC, USA

⁴The Sheikh Zayed Institute for Pediatric Surgical Innovation, Children’s National Health System, Washington, DC, USA

⁵Center for Cancer & Immunology Research, Children’s National Health System, Washington, DC, USA

⁶Center for Emerging Technologies in Immune Cell Therapy, Children’s National Health System, Washington, DC, USA

*Author for correspondence:

Tel.: +1 202 476 2046

CCruz@childrensnational.org

**Author for correspondence:

Tel.: +1 202 476 5290

RFernand@childrensnational.org

Future
Medicine

part of
fsg

and as multimodal imaging agents for *in vitro* [13,14] and *in vivo* [15] settings. The CTL possess the ability to traffic to and destroy EBV antigen-expressing target cells including EBV-infected B cells and EBV-positive cancer cells [16,17].

We present a generalizable scheme for robustly attaching PBNPs onto antigen-specific T cells (CTL:PBNP), and our work illustrates the physical and phenotypic properties of both the PBNPs and CTL within the CTL:PBNPs construct. In terms of functionality, we assess the ability of the CTL:PBNPs construct to both ablate (PBNP-specific) and lyse (CTL-specific) EBV antigen-expressing target cells. It is our hope that these results provide insight into the feasibility and functionality of a biohybrid CTL:PBNPs product to pave the way for future studies that demonstrate the potential of this novel nanoimmunotherapy for the treatment of infectious diseases and malignancies.

Materials & methods

Synthesis, biofunctionalization & analysis of the PBNPs

PBNPs were synthesized in ultrapure water at room temperature using a one-pot synthesis scheme, as previously described [13–15,18]. The resultant PBNPs were coated with filtered nonfluorescent- or AlexaFluor 488-conjugated avidin at a ratio of 0.1 mg avidin per 1 mg PBNPs via electrostatic self-assembly [13,15,19]. Following synthesis and coating, the size distributions and zeta potentials of the PBNPs or avidin-coated PBNPs were determined using light scattering techniques on a Zetasizer Nano ZS. To measure the absorption properties of the PBNPs and the PBNP-cell constructs, absorption scans in the visible-near infrared (NIR) wavelength range of 500–1100 nm were acquired on a Genesys 10S spectrophotometer (Appendix A; [Supplementary data](#) for details).

T cell & antigen-specific T-cell sources

Human Jurkat T cells were obtained from ATCC and were used to determine the feasibility of our nanoparticle attachment methodology. Human peripheral blood mononuclear cells (PBMC) were obtained from deidentified discarded blood products under an Institutional Review Board-approved protocol at Children's National Health System. PBMC from seven different donors were used to generate EBV antigen-expressing PHA blasts (target cells) and primary EBV antigen-specific T-cell lines (CTL) as previously described [16]. Briefly, the target PHA blasts were generated by pulsing with defined EBV peptides ([Supplementary data](#) for details). Hence these PHA blasts expressed defined EBV peptides and were not EBV-infected cells (Appendix A; [Supplementary data](#) for details).

Biofunctionalization, phenotyping & functional assessment of the T cells/CTL

Jurkat cells and CTL were biotinylated by incubation with a biotinylation reagent (sulfo-NHS-LC-biotin) [19] and were added to a solution of fluorescent avidin-coated PBNPs (containing 10^{-7} – 10^{-8} mg PBNPs/T cell). Using the robust interactions between avidin and biotin ($K_d = 10^{-15}$ M), we were able to obtain the conjugated nanoparticle-cell constructs [20]. The cells were then rinsed to remove unbound nanoparticles by centrifugation. Following this, the PBNPs were effectively attached onto the T cells and the biohybrid construct identified as CTL:PBNPs. The efficiency of the nanoparticle attachment was evaluated using confocal microscopy and flow cytometry. The phenotypes of uncoated and PBNP-coated T cells were characterized via flow cytometry using a panel of antibodies specific for T-cell markers. Functional assessment was evaluated using the CFSE flow cytometry-based proliferation assay, and cytokine production in response to antigen stimulation was analyzed by multiplex (Appendix A; [Supplementary data](#) for details).

Co-culture studies

To assess their cytolytic ability, CTL:PBNPs were added at a 2:1 ratio to fluorescently labeled target cells (primary PHA blasts pulsed with EBV peptides). The cells were cultured for 4–8 h after which PTT was administered. The co-cultures were established in a 96-well plate and individual wells were subject to PTT using an 808 nm NIR laser at 2.5 W/cm^2 for 10 min (Appendix A; [Supplementary data](#) for details). Target cell viability was determined from flow cytometry-based analysis, wherein an inclusive polygonal gating scheme including all fluorescently labeled target cells was used to account for potential shifts in cell populations due to changes in cell viability.

Results

Avidin–biotin conjugation enables successful attachment of PBNPs on CTL

In order to attach PBNPs to T cells, we took advantage of the robust avidin–biotin interactions by contacting avidin-coated PBNPs with biotinylated T cells ([Figure 1A](#)). Dynamic light scattering was used to measure the hydrodynamic diameters and surface charges (zeta potentials) of uncoated or avidin-coated PBNPs. Our synthesis and coating schemes yielded monodisperse size distributions of nanoparticles, in other words, mean hydrodynamic diameters ~ 80 – 90 nm, with polydispersity indices ~ 0.2 , and zeta potentials ~ -40 mV for both uncoated and avidin-coated PBNPs ([Supplementary Figure 1](#)). We utilized Jurkat T cells

Figure 1. Developing a schema for successful attachment of Prussian blue nanoparticles onto cytotoxic T lymphocytes. (A) We propose a schema by which fluorescent avidin-coated PBNPs are conjugated to the surface of biotinylated CTL. (B) Confocal microscopy images (20×) demonstrating successful conjugation of PBNPs (Alexafluor488+) onto Jurkat cells (DAPI+) taken immediately after cell-nanoparticle conjugation (Day 1); scale bar = 10 μm. (C & D) Flow cytometry was used to evaluate the persistence of the PBNP coating, as indicated by the AlexaFluor488+ population; cells were pre-gated as CD3⁺, n = 3. PBNP: Prussian blue nanoparticle.

to ascertain the feasibility of conjugating PBNPs to T cells to create the CTL:PBNPs constructs according to the scheme indicated (Figure 1A). Confocal

fluorescence microscopy confirmed the successful attachment of PBNPs onto T cells using this protocol (Figure 1B, right) as compared with uncoated T cells

Figure 2. Prussian blue nanoparticle conjugation does not impact cytotoxic T lymphocyte phenotype or function. (A & B) Representative histograms and aggregate data depicting proliferation of CFSE-labeled T cells following 24 h of antigen-specific stimulation. (C & D) Representative dot plots and aggregate data of CTL phenotype assayed by flow cytometry. PBNP: Prussian blue nanoparticle.

(Figure 1B, left), assessed immediately after the conjugation (Day 1). CTL:PBPNs constructs were also identified by flow cytometry – gating on CD3⁺ T cells that were also positive for Alexa Fluor 488 thereby confirming the successful conjugation of PBNPs onto CTL for up to 3 days (Figure 1C & D). The mean fluorescence intensity (MFI) of PBNPs attached to CTL nonsignificantly decreased over 3 days indicating that the quantity of nanoparticles per cell likely decreased over time (Supplementary Figure 2). These findings were corroborated with confocal microscopy conducted over 5 days, which demonstrated that the number of PBNPs per cell decreased by Day 3 (Supplementary Figure 2). This ‘dilution’ of surface-bound nanoparticles can be potentially attributed to the expansion of the CTL and/or internalization of the PBNPs by the cells. Our findings are consistent with an earlier study attaching nanoparticles onto the surface of cells [9], and illustrate the feasibility of the cell–nanoparticle conjugation schema of PBNPs attached to CTL based on avidin-biotin affinity.

CTL phenotype & function is not affected by PBNP conjugation

An important design consideration after achieving stable conjugation of nanoparticles onto cells is to ensure that both the nanoparticles and T cells retain their properties after incorporation into the biohybrid construct. As such, we sought to determine if the phenotype and functionality of antigen-specific T cells would be impaired by the presence of a nanoparticle coating. We observed that the proliferative capabilities of CTL alone and CTL:PBPNs constructs, stimulated by EBV peptide-loaded PHA blasts, were comparable, and both groups proliferated significantly more than the unstimulated CTL control ($p = 0.002$, $n = 7$; Figure 2A & B). Phenotypic analysis by flow cytometry demonstrated unaltered subsets of CD8⁺ and CD4⁺ T cells in CTL alone as compared with CTL:PBPNs (Figure 2C & D). Additional analysis of other subsets – CD45RA and CD45RO, as well as markers of exhaustion TIM3, LAG3, PD1 demonstrated no significant differences between CTL and CTL:PBPNs

Figure 2. Prussian blue nanoparticle conjugation does not impact cytotoxic T lymphocyte phenotype or function (cont.). (E) Aggregate results of cytokine production by stimulated CTL $\pm$ PBNP-coating at varying surface nanoparticle concentrations. (F) Viability of EBV+ target cells in response to cytotoxicity mediated by CTL $\pm$ PBNP-coating; $n = 7$. PBNP: Prussian blue nanoparticle.

(Supplementary Figure 3). In order to evaluate the functional capacity of uncoated and PBNP-coated CTL, we co-cultured CTL with EBV-positive target cells and measured cytokine concentrations in the supernatant as well as the viability of the target cells. Examination of cytokine production by stimulated CTL versus CTL conjugated with varying concentrations of PBNPs demonstrated no notable effect on cytokine production (Figure 2E). Flow cytometric analysis of target cell fluorescence within the designated conditions demonstrated a marked decrease in the viability of EBV antigen pulsed target cells following the treatment with EBV-specific CTL, which was maintained with the PBNP-coated EBV-CTL ($p = 0.15$, Figure 2F). Hence, our results demonstrate that EBV-specific CTL maintain their phenotype and antigen-dependent proliferative and functional ability while conjugated to nanoparticles within the CTL:PBNPs construct.

PBNPs conjugated onto CTL are functional as agents of PTT

In this study, PBNPs are primarily utilized as an agent of PTT. Therefore, it was essential to ensure that the fundamental properties of the PBNPs (i.e., their ability to absorb near infrared (NIR) light and ablate surrounding tissue) would not be impaired after attachment onto CTL. We first examined the Visible-NIR absorption spectra of CTL, PBNPs and CTL:PBNPs in the 500–1100 nm wavelength range to test whether the PBNPs retained their characteristic absorption peak in the range of 650–750 nm after conjugation to the CTL. We found that the PBNPs retained their Visible-NIR absorption characteristics even after conjugation to CTL (Figure 3A). Next we assessed the photothermal heating and cooling kinetics to assess the photothermal conversion efficiencies of CTL:PBNPs as compared with PBNPs alone. For

Figure 3. Prussian blue nanoparticles stably conjugated to cytotoxic T lymphocytes are functional as agents of photothermal therapy. (A) Vis-NIR spectra of CTL, PBNPs and the CTL:PBNPs construct: a peak in 680–720 nm indicates light absorption in the NIR range. (B) Temperature profile of the various groups in response to 10-min exposure to the NIR laser followed by cooling at room temperature. (C & D) Aggregate data depicting target cell viability and representative histograms (with live cell pre-gating) of labeled target cells in response to co-culture with CTL ± PBNPs ± laser; n = 7. CTL: Cytotoxic T lymphocyte; NIR: Near infrared; PBNP: Prussian blue nanoparticle.

this study, fluorescently labeled target cells (primary PHA blasts pulsed with EBV peptides) were co-cultured with CTL, PBNPs or CTL:PBNPs, and then

subjected to the NIR laser. Target cells co-incubated with either PBNPs or CTL:PBNPs and then subjected to the NIR laser exhibited equivalent photothermal

heating and cooling profiles, compared with target cells directly irradiated with the NIR laser and target cells co-cultured with CTL and then subjected to the NIR laser (Figure 3B & Supplementary Figure 4). Our findings demonstrate that PBNPs retain their photothermal conversion capabilities even after conjugation to CTL. Last, we determined whether the conjugation of PBNPs onto CTL would affect their ability to ablate primary target cells. Flow cytometric analysis of cell viability (fluorescently labeled primary PHA blasts pulsed with EBV peptides) illustrated a decrease in the viability of the target cells following treatment with CTL:PBNPs plus the NIR laser (i.e., target cells + CTL:PBNPs + LASER group; Figure 3C & D). In contrast, the negative controls of target cells treated with either the NIR laser alone or PBNPs alone did not impact target cell viability. Internal controls of target cells alone and target cells + DMSO were used to ensure cytotoxicity assay accuracy. Notably, our results showed that target cell viability was significantly decreased in the 'target cells + CTL:PBNPs + LASER' group compared with both 'target cells + CTL:PBNPs' and the 'Target Cells + PBNPs + LASER' group ($p < 0.05$ for both comparisons), which is likely attributed to the beneficial effects of combining the ablative capabilities of the PBNPs that are retained in the CTL:PBNPs constructs with the cytotoxic capabilities of the CTL themselves in the construct, compared with either killing modality by itself. Taken together, our results demonstrate that the PBNPs retain their intrinsic absorption properties, photothermal heating/cooling profiles and PTT capabilities even after conjugation to the CTL.

Discussion

Nanoparticles have been of considerable interest in medicine as a method of drug encapsulation and delivery, but their applicability for immunotherapy is not yet fully explored. In this work, we provide the crucial first steps toward generating a biohybrid nanoimmunotherapy using antigen-specific T cells as vehicles for PBNPs. We described a robust process for conjugating PBNPs onto primary EBV antigen-specific CTL and characterized the individual components, as well as the final CTL:PBNPs construct. Importantly, we demonstrated that the conjugation process maintained the individual functions of both the PBNPs and the CTL. This is critical for the newly proposed nanoimmunotherapy, as it builds upon the existing, well-established effector properties of both the nanoparticles and CTL. Our findings are consistent with other published reports combining nanoparticles with diverse immune cells [5,9,10,21]. In summary, this work serves as a proof-of-concept study demonstrating the feasibility of attaching of Prussian blue nanoparticles onto antigen-specific T cells. An important next step is the validation of our approach in animal models of infectious diseases and cancer. Our results also provide the basis for the future development of biohybrid platforms that combines the strengths of nanomedicine with that of cell-based immunotherapies, for the treatment of the aforementioned diseases.

Conclusion

We have described the successful conjugation of PBNPs onto donor-derived EBV antigen-specific CTL using a generalizable scheme involving biofunctional-

Executive summary

Synthesis of a nanoimmunotherapy comprising Prussian blue nanoparticles & antigen-specific cytotoxic T lymphocytes

- PBNPs utilized for their photothermal therapy (PTT) capabilities.
- Epstein-Barr virus (EBV) antigen-specific CTLs as a model for antigen-specific T cells, capable of specifically targeting cells expressing EBV antigens.

Prussian blue nanoparticles can be robustly conjugated onto the surface of cytotoxic T lymphocytes

- Prussian blue nanoparticles (PBNPs) biofunctionalized with avidin using electrostatic self-assembly.
- Cytotoxic T lymphocytes (CTLs) covalently biotinylated using an amine-reactive biotinylation reagent.
- PBNPs attached CTL using strong avidin-biotin interactions ($K_d = 10^{-15}$ M).

CTLs retain phenotype & function after PBNP-coating

- CTL proliferation and expression of cell surface markers not affected.
- CTL cytokine production and specificity for EBV antigen-expressing target cells (PHA blasts) maintained.

PBNPs retain their properties after attachment to CTLs

- Vis-NIR properties of PBNPs unaltered.
- Photothermal heating and cooling profiles of PBNPs unaltered.
- PBNPs retain their ability to be used for PTT.

Conclusion

- Proof-of-concept synthesis of a novel nanoimmunotherapy combining PBNPs with EBV-specific CTLs.
- CTLs retain their innate phenotype and function after incorporation into a cell-nanoparticle construct.
- PBNPs retain their intrinsic properties after attachment onto CTLs.

ization techniques (electrostatic self-assembly and cell surface biotinylation) and avidin–biotin interactions (Figure 1). Importantly, both the CTL and the PBNPs retained their intrinsic properties in the cell-nanoparticle construct. Specifically, PBNP-coated CTL retained their expression of T-cell-specific markers and their ability to proliferate, express cytokines and lyse EBV antigen-expressing target cells (PHA blasts) relative to uncoated CTL (Figure 2). The PBNPs retained their characteristic Vis-NIR absorption peak, their photothermal conversion capabilities, and their ability to be used for PTT of primary target cells (Figure 3). These studies suggest the potential use of our biohybrid CTL:PBNPs as a therapeutic for the treatment of cancer.

Future perspective

We envision the future impact of our nanoimmunotherapy in the treatment of cancer. One of the biggest limitations of nanoparticles in cancer therapy is poor tumor targeting and uptake. In the case of T-cell therapies, one of the biggest limitations is the immune-suppressive environment maintained by malignancies [22–25]. We foresee that our nanoimmunotherapy could potentially overcome these limitations with a two-pronged attack: First, therapeutic immune cells will localize to the tumor antigen and exert a cytotoxic effect on tumor cells. Second, PBNPs, having homed to the tumor by way of their vehicle T cells, can be activated with an NIR laser (either intraoperatively for deeper tumors or through the skin for superficial tumors), providing additional cytotoxic effect by PTT plus potential antitumor immunostimulatory effects elicited by PTT. These antitumor effects can be further leveraged with adjuvants attached to the PBNPs, for example, antibodies targeting either immunosuppressive pathways or activating receptors to increase T-cell activation in the hostile tumor environment.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

- Schutz CA, Juillerat-Jeanneret L, Mueller H, Lynch I, Riediker M. Therapeutic nanoparticles in clinics and under clinical evaluation. *Nanomedicine (Lond.)* 8(3), 449–467 (2013).
- Rink JS, Plebanek MP, Tripathy S, Thaxton CS. Update on current and potential nanoparticle cancer therapies. *Curr. Opin. Oncol.* 25(6), 646–651 (2013).
- Swartz MA, Hirose S, Hubbell JA. Engineering approaches to immunotherapy. *Sci. Transl. Med.* 4(148), 148rv149 (2012).

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0160

Acknowledgements

The authors acknowledge the Sheikh Zayed Institute for Pediatric Surgical Innovation at Children's National Health System and the Institute of Biomedical Sciences at George Washington University, where RAB is a doctoral student.

Disclaimer

The contents of this report contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Center for Advancing Translational Sciences or the National Institutes of Health.

Financial & competing interests disclosure

This work was also supported by Awards UL1TR000075 and KL2TR000076 from the NIH National Center for Advancing Translational Sciences. This work is covered by pending patents. R Fernandes is listed as an inventor on a pending patent US20140271487 A1. RA Burga, CM Bollard, CRY Cruz and R Fernandes are listed as inventors on a United States provisional patent application (application number in process). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

- Goldberg MS. Immunoengineering: how nanotechnology can enhance cancer immunotherapy. *Cell* 161(2), 201–204 (2015).
- Provides an important overview of how nanotechnology can be combined with immunotherapy for cancer therapy.
- Bear AS, Kennedy LC, Young JK *et al.* Elimination of metastatic melanoma using gold nanoshell-enabled photothermal therapy and adoptive T cell transfer. *PLoS ONE* 8(7), e69073 (2013).
- Wang C, Xu L, Liang C, Xiang J, Peng R, Liu Z. Immunological responses triggered by photothermal therapy with carbon nanotubes in combination with anti-CTLA-4 therapy to inhibit cancer metastasis. *Adv. Mater.* 26(48), 8154–8162 (2014).

- 7 Zhang P, Chiu YC, Tostanoski LH, Jewell CM. Polyelectrolyte multilayers assembled entirely from immune signals on gold nanoparticle templates promote antigen-specific T cell response. *ACS Nano* 9(6), 6465–6477 (2015).
- 8 Flynn ER, Bryant HC, Bergemann C, Larson RS, Lovato D, Sergatskov DA. Use of a SQUID array to detect T-cells with magnetic nanoparticles in determining transplant rejection. *J. Magn. Magn. Mater.* 311(1), 429–435 (2007).
- 9 Stephan MT, Moon JJ, Um SH, Bershteyn A, Irvine DJ. Therapeutic cell engineering with surface-conjugated synthetic nanoparticles. *Nat. Med.* 16(9), 1035–1041 (2010).
- **Demonstrates how cell-nanoparticle constructs can be used for enhanced cell therapy *in vivo***
- 10 Swiston AJ, Gilbert JB, Irvine DJ, Cohen RE, Rubner MF. Freely suspended cellular “backpacks” lead to cell aggregate self-assembly. *Biomacromolecules* 11(7), 1826–1832 (2010).
- 11 Polak R, Lim RM, Beppu MM, Pitombo RN, Cohen RE, Rubner MF. Liposome-loaded cell backpacks. *Adv. Healthc. Mater.* 4(18), 2832–2841 (2015).
- 12 Hoffman HA, Chakrabarti L, Dumont MF, Sandler AD, Fernandes R. Prussian blue nanoparticles for laser-induced photothermal therapy of tumors. *RSC Adv.* 4(56), 29729–29734 (2014).
- **Demonstrates the synthesis and use of Prussian blue nanoparticles for tumor therapy *in vivo*.**
- 13 Dumont MF, Hoffman HA, Yoon PR *et al.* Biofunctionalized gadolinium-containing prussian blue nanoparticles as multimodal molecular imaging agents. *Bioconjug. Chem.* 25(1), 129–137 (2014).
- 14 Vojtech JM, Cano-Mejia J, Dumont MF, Sze RW, Fernandes R. Biofunctionalized prussian blue nanoparticles for multimodal molecular imaging applications. *J. Vis. Exp.* (98), e52621 (2015).
- 15 Dumont MF, Yadavilli S, Sze RW, Nazarian J, Fernandes R. Manganese-containing Prussian blue nanoparticles for imaging of pediatric brain tumors. *Int. J. Nanomedicine* 9, 2581–2595 (2014).
- 16 Bollard CM. Improving T-cell therapy for Epstein–Barr virus lymphoproliferative disorders. *J. Clin. Oncol.* 31(1), 5–7 (2013).
- 17 Bollard CM, Aguilar L, Straathof KC *et al.* Cytotoxic T lymphocyte therapy for Epstein–Barr virus+ Hodgkin’s disease. *J. Exp. Med.* 200(12), 1623–1633 (2004).
- **Describes the generation of EBV antigen-specific CTL.**
- 18 Volz HG. Pigments, Inorganic. *Ullmann’s Encyclopedia of Industrial Chemistry*. Wiley (2006).
- 19 Jaiswal JK, Mattoussi H, Mauro JM, Simon SM. Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat. Biotechnol.* 21(1), 47–51 (2003).
- **Provides the scheme for robustly bioconjugating nanoparticles onto cells.**
- 20 Goldman ER, Balighian ED, Mattoussi H *et al.* Avidin: a natural bridge for quantum dot-antibody conjugates. *J. Am. Chem. Soc.* 124(22), 6378–6382 (2002).
- 21 Choi MR, Bardhan R, Stanton-Maxey KJ *et al.* Delivery of nanoparticles to brain metastases of breast cancer using a cellular Trojan horse. *Cancer Nanotechnol.* 3(1–6), 47–54 (2012).
- 22 Arina A, Bronte V. Myeloid-derived suppressor cell impact on endogenous and adoptively transferred T cells. *Curr. Opin. Immunol.* 33, 120–125 (2015).
- 23 Arina A, Corrales L, Bronte V. Enhancing T cell therapy by overcoming the immunosuppressive tumor microenvironment. *Semin. Immunol.* 28(1), 54–63 (2016).
- 24 Munn DH, Bronte V. Immune suppressive mechanisms in the tumor microenvironment. *Curr. Opin. Immunol.* 39, 1–6 (2016).
- 25 Burga RA, Thorn M, Point GR *et al.* Liver myeloid-derived suppressor cells expand in response to liver metastases in mice and inhibit the anti-tumor efficacy of anti-CEA CAR-T. *Cancer Immunol. Immunother.* 64(7), 817–829 (2015).