Non-invasive early detection of acute transplant rejection via nanosensors of granzyme B activity

Quoc D. Mac1,8, Dave V. Mathews2,8, Justin A. Kahla1, Claire M. Stoffers1, Olivia M. Delmas1, Brandon Alexander Holt1, Andrew B. Adams2,3* and Gabriel A. Kwong1,4,5,6,7*

The early detection of the onset of transplant rejection is critical for managing immunosuppression and the long-term survival of patients. The diagnostic gold standard for detecting transplant rejection involves a core biopsy, which is invasive, has limited predictive power and carries a morbidity risk. Here, we show that nanoparticles conjugated with a peptide substrate specific for the serine protease granzyme B, which is produced by recipient T cells during the onset of acute cellular rejection, can serve as a non-invasive biomarker of early rejection. When administered systemically in mouse models of skin graft rejection, these nanosensors preferentially accumulate in allograft tissue, where they are cleaved by granzyme B, releasing a fluorescent reporter that filters into the recipient's urine. Urinalysis then discriminates the onset of rejection with high sensitivity and specificity before features of rejection are apparent in grafted tissues. Moreover, in mice treated with subtherapeutic levels of immunosuppressive drugs, the reporter signals in urine can be detected before graft failure. This method may enable routine monitoring of allograft status without the need for biopsies.

Organ transplantation remains the single most effective treatment for end-stage organ failure, and early detection of transplant rejection is critical for managing immunosuppression and the long-term survival of recipients1–5. During acute cellular rejection (ACR), graft damage is mediated by recipient cytotoxic CD8+ T cells that are activated by alloantigens displayed by antigen-presenting cells (APC) and target allogeneic cells for killing6–8. Although ACR episodes may appear at any time during the life of the graft, even years after immunological quiescence9, ACR can be effectively treated with anti-rejection drugs that target T cells (for example, cyclosporine, thymoglobulin or anti-CD3 antibodies). Therefore the ability to measure the level of anti-graft T cell responses at an early stage of ACR plays an indispensable role in managing long-term graft health and function9. Currently, the gold standard for diagnosing ACR is core tissue biopsy, but this procedure is invasive, subject to sampling error (the tissue specimen typically represents approximately only 1/10,000th the volume of the organ), and associated with potential patient morbidity10. Noninvasive approaches include measuring biomarkers that indicate organ dysfunction, such as blood urea nitrogen (BUN) and serum creatinine for kidney allografts11–13, or biomarkers associated with allograft cell death, such as cell-free donor-derived DNA from the blood of heart transplant patients14. These biomarkers indicate graft health at a stage of rejection when organ dysfunction or damage is clinically apparent.

The mechanism by which activated cytotoxic T cells engage and kill target cells is well studied and involves the release of cytolytic granules containing perforin, which forms pores in target cell membranes, and granzymes, which are serine proteases, to trigger apoptosis by cleavage of caspases or activation of mitochondria and DNA damage pathways15,16. Early-onset ACR or subclinical ACR, defined according to Banff stages IA and IB of T-cell-mediated rejection (TCMR), have been directly shown to correlate to increased granzyme B (GzmB) expression17–19. In renal allografts, the level of GzmB+ lymphocytes are significantly higher in stages IA and IB compared to control biopsies17–19, and predict rapid progression to severe ACR (TCMR grade II or higher)20. Clinical studies focused on quantifying RNA transcripts showed that elevated GzmB levels in blood or urine are correlated to early ACR (grade IB or lower) and precede clinical diagnosis of ACR in renal and islet transplant patients21–23. Importantly, the activity of GzmB is also regulated by tissues locally; increased expression of the endogenous GzmB inhibitor serpin protease inhibitor 9 (PI-9)20 was reported to be a potential mechanism for stable renal function in patients with subclinical ACR21 by inhibiting the ability of GzmB to trigger apoptosis in target cells. These clinical studies provide direct support for targeting GzmB as an early indicator of ACR and highlight the need to develop new methods to measure the activity of GzmB within the context of the local tissue microenvironment.

A promising approach to noninvasively measure in vivo protease activity is the design and administration of engineered agents to investigate diseased tissue. These include activity-based imaging probes that emit fluorescent signals upon cleavage24, or selectively bind to the active form of proteases25. Recently, this approach was employed for in vivo imaging of GzmB activity during experimental myocarditis and monitoring the treatment efficacy of cancer immunotherapy with positron emission tomography (PET) probes that irreversibly bind to GzmB26–28. Activity-based approaches using fluorescent reporters are limited to superficial sites due to tissue scattering, but can be used in deeper tissues with invasive procedures, such as during tumor resection24,28. Here we engineer GzmB nanosensors that accumulate in allograft tissue to sense anti-graft T cell activity by producing a signal that is shed into urine for noninvasive detection (Fig. 1). Our technology builds on our work
on synthetic biomarkers, which leverage the catalytic activity of proteases to amplify detection signals, as well as the enrichment of detection signals into urine, to allow ultrasensitive detection of early-stage disease. In preclinical skin graft models of rejection, our GzmB activity nanosensors allow noninvasive discrimination of early ACR, and indicate graft failure in recipients under subtherapeutic immunosuppression.

Results

Engineering activity nanosensors against granzyme B. Our activity nanosensors are formulated by conjugating GzmB peptide substrates to the surface of a nanoparticle scaffold (Fig. 2a). A nanoparticle carrier extends the circulation half-life of GzmB peptide substrates (Supplementary Fig. 1), which would otherwise be rapidly filtered into urine, and increases peptide accumulation in tissues by passive diffusion from the circulating vasculature. Protease cleavage of the nanosensor by GzmB triggers a pharmacokinetic switch by releasing peptide fragments locally, which are then remotely cleared into urine for noninvasive detection. Here we used iron oxide nanoparticles (IONPs) as they are tolerated well and are FDA-approved for clinical use as anaemia therapies, contrast agents and in thermal ablation. To increase biocompatibility and circulation half-life, we decorated IONPs synthesized in house with polyethylene glycol (PEG) to reduce nanoparticle uptake by the reticuloendothelial system (RES). With moderate PEGylation (20:1 PEG to IONP ratio), the average hydrodynamic diameter of our IONPs was ~47 nm while the circulation half-life was increased to ~3 h in vivo (Supplementary Fig. 2). These values are consistent with previously reported values for clinically approved IONPs.

To identify peptide substrates that are sensitive to cleavage by recombinant GzmB, we pooled 13 candidate substrates from published literature and calculated a $k_{cat}/K_m$ value of $1.09 \times 10^4 \text{M}^{-1} \text{s}^{-1}$. This value was similar in magnitude to reported values of GzmB cleavage of free substrates (Fig. 2b) and showed that co-presentation of substrates and PEG molecules (Supplementary Fig. 4) on the surface of IONPs did not sterically hinder GzmB cleavage. To assess substrate specificity for GzmB, we exposed our probes to coagulation and complement proteases, as they are ubiquitous and found at high concentrations in circulating blood. Using saline samples spiked with recombinant proteases (Fig. 2c), plasma samples spiked with Ca$^{2+}$ to trigger coagulation (Fig. 2d and Supplementary Fig. 5), or heat aggregated γ-globulin (HAGG) to trigger complement (Fig. 2e,f), we did not observe detectable increases in fluorescence that would indicate cross-cutting of our probes. By contrast, our probes were markedly activated in all samples that contained recombinant GzmB. To further test the biocompatibility of our nanosensors, we assessed the level of membrane attack complex (MAC) in serum samples to quantify potential complement activation from a foreign surface. We found significant increases in MAC levels in samples incubated with HAGG, but by contrast, no elevation in MAC formation in the presence of our probes ($P < 0.001, n = 3$, Fig. 2f). Taken together, our activity nanosensors are sensitive to GzmB cleavage, are not cross-cut by coagulation and complement cascades, and do not promote complement activity.

GzmB activity nanosensors detect alloreactive T cell killing. During target cell killing, an immune synapse is formed between cytotoxic T lymphocytes (CTLs) and target cells, and cytotoxic granules are directionally released into the synaptic cleft. This mechanism is thought to increase the local concentration of effector molecules to enhance target cell entry and killing, as well as to decrease off-target cytotoxicity by limiting exposure of bystander cells to effector molecules. As self-protective mechanisms, CTLs express cell-surface cathepsins and intracellular serpin PI-9 to proteolytically degrade granzymes or inhibit their cleavage activity. Although the release of effector molecules is directed to the
immune synapse, extracellular GzmB can be readily detected using standardized assays such as ELISpot. Moreover, cell-free GzmB levels have been used to monitor cytotoxic activity in vivo, such as with cancer and rheumatoid arthritis. Because our nanosensors are formulated to probe extracellular protease activity, we sought to quantify GzmB expression during T cell killing in both intracellular and extracellular spaces, and assess the ability of our nanosensors to quantify GzmB expression during T cell killing in both intracellular and extracellular spaces, and assess the ability of our nanosensors to detect anti-graft cytotoxicity ex vivo.

To validate that the nanosensors are sensitive to physiologically relevant concentrations of GzmB, we used a T cell killing assay composed of transgenic OT1 T cells, which recognize the peptide epitope SINFEKL from chicken ovalbumin (OVA), and target EG7-OVA or EL4 cells that express or lack the OVA antigen, respectively. We first verified intracellular expression of GzmB in activated OT1 T cells co-cultured with EG7-OVA target cells compared to EL4 controls (Supplementary Fig. 6a,b). To confirm protease activity of GzmB, we used commercial fluorogenic probes that produce fluorescent signals after intracellular cleavage by GzmB in target cells. Although GzmB activity was not significantly elevated in EL4 target cells, significant GzmB activity was observed in EG7-OVA target cells, although GzmB activity was not significantly elevated in EL4 target cells, significant GzmB activity was observed in EG7-OVA target cells, with BALB/c splenocytes and nanosensors resulted in significant increases in protease activity as monitored by sample fluorescence, which was consistent with the level of extracellular GzmB as quantified by ELISA (Fig. 3a–c). To quantify the amount of GzmB secreted into extracellular spaces, we analysed co-culture supernatants by ELISA and detected an approximately 10-fold increase in GzmB concentrations (~1.7 pM (~50 pg ml⁻¹) which indicated that significant amounts of GzmB were released into the extracellular space. Under these co-culture parameters, we incubated fluorescently labelled nanosensors with conditioned supernatant and detected significant increases in initial cleavage velocities (V₀) at an OT1: EG7-OVA ratio of 10, which was consistent with the level of extracellular GzmB as quantified by ELISA (Fig. 3d–f).

To establish the sensitivity of our nanosensors against alloreactive T cells, we collected splenocytes from donor C57BL/6 mice. Co-incubation of harvested allograft cells transplanted with skin allografts from major histocompatibility complex (MHC) mismatched BALB/c donor mice or isografts from donor C57BL/6 mice. Co-incubation of harvested allograft cells transplanted with skin allografts from major histocompatibility complex (MHC) mismatched BALB/c donor mice or isografts from BALB/c donors resulted in significant increases in GzmB activity as monitored by sample fluorescence, which was not detected in samples co-cultured with T cells harvested from isograft mice (Fig. 3g,h). These results showed that GzmB activity nanosensors detect alloreactive T cell killing activity.
Fig. 3 | Sensing GzmB activity during alloreactive T cell killing. a, After upregulating expression, activated OT1 T cells secrete GzmB, which enters and mediates apoptosis of EG7-OVA target cells. b, Flow cytometry plots of GzmB activity within EG7-OVA and EL4 target cells after co-cultured with OTI T cells at various T cell to target cell ratios. c, Quantified plot of flow analysis showing percent of target EG7-OVA and EL4 cells with intracellular GzmB activity (one-way ANOVA with Turkey’s post-test and correction for multiple comparisons, ***P < 0.001, n = 3). Central values = means, and error bars = s.e.m. d, ELISA assay measuring levels of GzmB in co-culture supernatants of OTI T cells with EG7-OVA or EL4 target cells at different T cell to target cell ratios (one-way ANOVA and Turkey’s post-test and correction for multiple comparisons, ****P < 0.0001, n = 3; nd = not detected). Central values = means, and error bars = s.e.m. e, T cell activity assays showing normalized fluorescence of activity nanosensors in co-culture supernatants of OTI T cells with EG7-OVA or EL4 target cells. This experiment was repeated independently 5 times with similar results. f, Quantified plot of T cell activity assays showing fitted values of initial cleavage velocities (one-way ANOVA and Turkey’s post-test and correction for multiple comparisons, ***P < 0.001, n = 3). Central values = means, and error bars = s.e.m. g, Activity nanosensors sense GzmB secreted during alloreactive T cell killing. h, T cell activity assays showing normalized fluorescence of activity nanosensors in co-culture supernatants of T cells isolated from spleen (SL) or draining lymph nodes (dLN) of skin graft mice with target cells from BALB/c donor mice. This experiment was repeated independently 3 times with similar results.
signal increase over skin isograft ($P < 0.01, n = 5$) and was statistically equivalent by POD 9, which we attributed to tissue necrosis, scab formation and vascular occlusion characteristic of late-stage rejection of allografts (Fig. 4a,b and Supplementary Fig. 7). To assess biodistribution, we found higher levels of IONPs in organs that preferentially accumulate and activate in allograft cohorts of mice (Supplementary Fig. 8). These studies were performed post-mortem and did not allow direct quantification of IONPs in the bladder due to spontaneous voiding of urine resulting from the relaxation of urethral sphincter muscles. Therefore, we used live whole-animal fluorescent imaging and found no accumulation of IONPs in the bladders of mice, which is consistent with the kidney size filtration limit (~5 nm) previously shown for inorganic nanoparticles (Supplementary Fig. 9a–c). To examine peptide pharmacokinetics, we administered free fluorophore-labelled GzmB substrates and found significant increases in perfusion in allograft tissue compared to isograft controls ($P < 0.05, n = 5$ for PODs 1, 3, 7, 9 and $n = 6$ for POD 5). Fluorescent signals from different days were normalized by signals from healthy skin. Central values = means, and error bars = s.e.m. c. A schematic of an NIR activatable probe that is conjugated with GzmB substrates containing internal fluorophores and terminal quenchers. After cleavage, the quencher is liberated to allow the nanoparticle to fluoresce. d. Fluorescence of major organs harvested from allograft and isograft mice dosed with the NIR activatable probe. (two-way ANOVA and Sidak post-test and correction for multiple comparisons, $***P < 0.0001, n = 6$). Fluorescent signals were normalized by signals from isograft mice. Inset: NIR fluorescent image of representative excised skin grafts. Central values = means, and error bars = s.e.m. e. Mice bearing skin isograft or isograft or isograft are dosed with activity nanosensors with terminal fluorophores, which can trigger a urine pharmacokinetic switch upon activation by GzmB during alloreactive T cell killing. f. Fluorescence of tissue homogenates from allograft and isograft mice after administration of activity nanosensors (two-way ANOVA and Sidak post-test and correction for multiple comparisons, $P < 0.05, n = 4$ isograft mice and $n = 6$ allograft mice). Fluorescent signals were normalized by signals from isograft mice. Central values = means, and error bars = s.e.m. g. Whole-body NIR fluorescent image after administration of activity nanosensors showing fluorescent signals from the bladders of allograft-bearing mice. This experiment was repeated independently 3 times with similar results.

Fig. 4 | Granzyme B activity during ACR triggers a urine pharmacokinetic switch. a. Mice bearing both isograft (Iso) and allograft (Allo) tissues are dosed with surface-labelled nanosensors for allograft accumulation and biodistribution studies. b. Top: photographs of mice bearing dual skin grafts and the corresponding near-infrared (NIR) fluorescent images. Bottom: quantified fluorescent intensities of excised skin grafts (two-way ANOVA and Sidak post-test and correction for multiple comparisons, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 5$ for PODs 1, 3, 7, 9 and $n = 6$ for POD 5). Fluorescent signals from being washed away and allows tissue localization of fluorescent IONPs. We first verified probe activation in vitro by recombinant GzmB (Supplementary Fig. 10a–c), and in vivo, found that probe activation was significantly increased in allograft skin tissue compared to isograft controls (Fig. 4d and Supplementary Fig. 11). To visualize peptide cleavage and trafficking of fluorescent reporter fragments into urine, we infused IONPs conjugated with homochanged GzmB substrates (Supplementary Fig. 12) and analysed major organs by fluorescent imaging (Fig. 4e). In allograft mice, we found 3-fold higher fluorescent signals in kidneys compared to isograft mice ($P < 0.0001, n = 4–5$, Fig. 4f and Supplementary Fig. 13), which we attributed to active renal filtration of cleaved peptide fragments. This observation was further supported in live animal imaging, where we observed increases in fluorescent signals in bladders of allograft mice (Fig. 4g). Collectively, our data showed that GzmB activity nanosensors preferentially accumulate and activate in allograft tissue during rejection, and after cleavage, peptide fragments are efficiently cleared into host urine.

Noninvasive and early detection of ACR. Histological criteria for staging severity of ACR include features such as tissue damage and the presence of apoptotic cells, which are downstream effects of anti-graft T cell responses. Activity measurements of proteases that drive disease pathology have the potential to be early biomarkers and anticipate disease trajectory, such as using matrix
metalloproteinase (MMP) activity to predict liver fibrosis progression and regression\textsuperscript{27,28}. We therefore investigated the potential of using GzmB activity nanosensors for the early detection of ACR (Fig. 5a). To quantify skin graft health and rejection kinetics, we assigned a score of 4 for healthy allografts, a score of 0 for full rejection, and intermediate scores based on features such as the ratio of viable to necrotic skin and the presence of ulcerations or scabs. According to these metrics, graft scores began to significantly decrease at day 9 after transplant (2.5 vs 3.9, \(P<0.0001\), n = 8). We detected an increase in the frequency of GzmB-expressing CD8\textsuperscript{T} cells from spleens and draining lymph nodes at days 5, 7 and 9. While GzmB levels at day 5 were unchanged compared to controls, we detected an increase in the frequency of GzmB-expressing CD8\textsuperscript{T} cells in allograft animals by day 7 (Supplementary Fig. 15) when graft scores remained statistically equivalent between allograft and isograft tissues. To support our findings, we analysed graft tissue on day 7 by immunohistochemistry and found significant increases in both graft-infiltrating CD8\textsuperscript{T} cells and GzmB expression (Fig. 5d–g). Taken together, our data provided evidence that GzmB levels were significantly upregulated in allograft tissue at the onset of acute rejection.

To determine conditions for serial monitoring, we first evaluated the immunogenicity of our formulation and found that GzmB peptides or nanosensors do not induce neutralizing antibodies when co-injected with or without complete Freund’s adjuvant (Supplementary Fig. 16). Moreover, we analysed residual urinary fluorescence from a single injection and found full clearance within 2 days after administration (Supplementary Fig. 17). Based on these results, we sought to determine how early our system can detect the onset of ACR by administering nanosensors and performing urinalysis in transplanted mice before transplantation (POD –4) and on early PODs at 2-day intervals (PODs 3, 5 and 7). Using pre-graft urine signals as a baseline for recipients, we did not detect significant elevations in post-graft urine signals from naive, isograft and CD8\textsuperscript{T}-depleted control cohorts of mice (Fig. 5h and Supplementary Fig. 18a,b). By contrast, post-graft urine signals from allograft recipients were sig-
significantly elevated as early as POD 5 \((P < 0.01, n = 6)\) and further increased by POD 7 \((P < 0.0001, n = 6, \text{Fig. 5b})\), detecting GzmB activity several days before graft scores were statistically significant between allografts and isografts (Fig. 5c). To assess sensitivity and specificity, we analysed urine signals by receiver-operating-characteristic (ROC) analysis and quantified an increase in AUROC from 0.67 to 0.98 by POD 7 (95% confidence interval of 0.94 to 1.03). Altogether, these results illustrate that systemic administration of GzmB activity nanosensors in allograft recipients produce urine signals that classify ACR with high sensitivity and specificity.

The use of immunosuppressive drugs as maintenance therapy has significantly reduced the rates of ACR; however, rejection episodes may occur despite ongoing immunosuppression, or in patients treated with subtherapeutic doses\(^{64,65}\). To assess the sensitivity of this approach to detect T cell activity under immunosuppression, we performed urinalysis of allograft-bearing mice before (POD 4) and after transplantation (PODs 7 and 12) (one-way ANOVA with Dunnett post-test, \(P < 0.01\)). Urine signals were quantified as percent injected dose before being normalized by pre-transplant signals. Fold increase in urine signals relative to pre-transplant signals in tacrolimus-treated allograft-bearing mice before (POD 4) and after transplantation (PODs 7 and 12). Urine signals from treated mice were blunted compared to untreated mice (Fig. 6c). These results showed that our urine measurements are sensitive to GzmB activity under subtherapeutic immunosuppression and provide an early indicator of eventual graft failure.

**Discussion**

Invasive core biopsy is considered the gold standard for diagnosing ACR; however, it is associated with significant patient morbidity and sampling variability, and provides only a static snapshot of anti-graft activity\(^6\). Here, we developed GzmB activity nanosensors, consisting of an IONP core decorated with peptide substrates, to sense CD8\(^+\) T cell killing. In a skin graft mouse model of ACR, these nanosensors accumulate in allograft tissue and are cleaved locally by GzmB, which then triggers a pharmacokinetic switch to enable detection of peptide fragments in the urine. Our nanosensors noninvasively detect the onset of ACR with high sensitivity and specificity, and in allograft mice under subtherapeutic immunosuppression, produce urine signals that precede eventual graft failure.

The use of a nanoparticle carrier increases circulation half-life of substrate peptides, which are otherwise cleared from the body by renal filtration within minutes after intravenous administration\(^14,15\). Without conjugating peptides to a nanoscale scaffold larger than the glomerulus pore size of \(~5\) nm\(^6\), urine samples would otherwise be confounded with uncleaved peptide substrates. We selected IONPs because they are FDA-approved and well-tolerated in humans\(^16\), but alternative formulations with different carriers (for example, PEG or dextran) can be used to tune pharmacokinetics\(^6\). Here we leveraged graft inflammation during early ACR\(^6,14,16\) to passively deliver nanosensors. Because skin transplants are initially avascular, we anticipated that passive targeting may be enhanced for vascularized solid transplants (for example, kidney, liver or heart), particularly for organs with porous, fenestrated endothelium such as the liver. Given the wealth of targeting and delivery strategies in nanomedicine, this system will be amenable to functionalization with organ-specific or inflammation-targeting ligands\(^6,16\) to direct delivery to specific anatomical sites or enhance diagnostic signals\(^6\).

Our study focused on GzmB activity as an early biomarker for ACR. Clinical studies have shown that increased expression of GzmB is found in allograft tissue during Banff TCRM IA and IB, precedes progression to grade II or higher, and has the potential to predict graft outcomes\(^7,14,15,17,19,66\). To measure protease activity in vivo, existing activity-based sensors are designed to produce a localized fluorescent signal, which is subject to tissue scattering and washout by blood, which attenuates the strength of the detection signal\(^14,67\). By contrast, our technology is based on local cleavage and remote detection of cleaved reporters enriched in urine, which amplifies detection signals to increase the sensitivity for early-stage analysis. Noninvasive biomarkers that rely on shed molecules or the contents of dead cells\(^33\) experience significant dilution in blood and may be limited for early-stage detection\(^7,72\). While the performance of this method for ACR detection in a preclinical setting (AUROC = 0.98) compares favourably to other biomarkers, such as serum creatinine (0.63), NGAL (0.6–0.9), KIM-1 (0.8) and cystatin C (0.64–0.82)\(^7,14,28,29\), the translation of this approach will require rigorous evaluation in humans and guidance by regulatory and community qualifications\(^32\).

The use of CNIs such as tacrolimus is closely monitored in patients to maintain graft tolerance and target blood concentrations\(^34\). In our study, the tacrolimus dose was subtherapeutic despite administration at doses (2 mg kg\(^{-1}\)) 20–200-fold higher compared to those given to human patients, and did not prevent eventual graft loss in mice. This discrepancy in dose results from the use of...
MHC-mismatched donors and recipients in skin graft models to allow ACR to occur within a reasonable experimental window (~7–10 days). In humans, donor and recipient MHC matching allows the use of lower effective concentrations of CNIs to induce and maintain graft tolerance. Our study showed that urine measurements of GzmB activity anticipated eventual graft failure in mice under subtherapeutic immunosuppression, which may allow for dose adjustment or additional therapeutic interventions in patients.

In the future, this technology will benefit by expanding our single GzmB probe into a family of nanosensors designed to sense different proteases, which can be accomplished by the use of mass barcodes that uniquely label each peptide substrate for multiplexed quantification by mass spectrometry. We anticipate that a family of activity sensors against a panel of proteases (for example, T cell, fibrosis and viral proteases) will allow staging of ACR and discrimination between different injuries that likewise depend on CD8+ T cell cytotoxicity. These include the ability to differentially diagnose anti-graft from anti-viral activity (for example, BK, CMV or HCV) that occurs with high prevalence in transplant patients. The expanded library may also include proteases from the complement cascade to allow monitoring of antibody-mediated rejection (AMR), which is responsible for the majority of long-term graft loss, or organ-specific proteases (for example liver protease hepsin) for signal normalization. These improvements may provide the capacity to differentiate ACR from AMR with high diagnostic power, and resolve anti-graft activity into the relative contributions of each mechanism to personalize management of transplant recipients.

Methods

Animals. Six- to eight-week-old male mice were used at the outset of all experiments. OT1 transgenic mice (C57BL/6-Tg( Erga tcrb1)100Mbj)) were purchased from Jackson Laboratories. C57BL/6 and BALB/c mice for skin graft experiments and CFU control mice were purchased from Charles River Laboratories. All animal protocols were approved by Georgia Tech IACUC (protocol no. A140711) and Emory University IACUC (protocol no. DAR: 202279-082117GN). All authors have complied with relevant ethical regulations while conducting this study.

Nanosensor synthesis and characterization. Aminated IONPs were synthesized in-house according to the published protocol. FITC-labelled GzmB substrate peptides ((FITC)AIEFDSGc; lower case letters = d-form amino acids) were synthesized by Tusfts University Core Facility and used for in vivo formulation. FITC-labelled GzmB substrate peptides with internal quencher (5-FAM) aEFDGK(CPQQ)kkc were synthesized by CPC Scientific and used for all in vitro activity assays. Aminated IONPs were first reacted to the heterobifunctional crosslinker succinimidyl isoaoacetate (SIA; Thermo) for 2 h at room temperature (RT), and excess SIA was removed by buffer exchange using the Amicon spin filter (30 kDa, Millipore). Sulhydrl-terminated peptides and polyethylene glycol (PEG; LaysanoBio, M-Sh-20K) were mixed with NP-SIA (90:20:1 molar ratio) and reacted overnight in RT in the dark to obtain fully conjugated activity nanosensors. Activity nanosensors were purified on a Superdex (90:20:1 molar ratio) and reacted overnight in RT in the dark to obtain fully conjugated activity nanosensors. Activity nanosensors were purified on a Superdex 200 Increase 10–300 GL column using the AKTA Pure FPLC System (GE Healthcare Care). Ratios of FITC per IONP were determined using absorbance of FITC (488 nm, e = 78,000 cm−1 M−1) and IONP (400 nm, e = 2.07 × 108 cm−1 M−1) as measured with a Cytation 5 plate reader (BioTek). At this conjugation condition, we co-activated OT1 CD8+ T cells with EL4 and GzmB-targeting activity nanosensors.

In vitro protease cleavage assays. Activity nanosensors (6 nM by NP, 300 nM by peptide) were incubated in PBS + 1% bovine serum albumin (BSA; Sigma) at 37 °C with murine granzyme B (0.17 μM; Biozoo). GzmB substrate peptides were isolated from OT1 spleenocytes (Jackson Labs) by MACS using CD8a microbeads (Miltenyi). Cells were activated by seeding in 96-well plates pre-coated with anti-mouse CD3e (1 μg ml−1 working concentration; clone: 145–2C11, BD) and anti-mouse CD28 (2 μg ml−1 working concentration; clone: 37.5L, BD) at 2 × 10^5 cells ml−1 in RPMI 1640 supplemented with 10% FBS, 100 U ml−1 penicillin-streptomycin, 1X non-essential amino acids (Gibco), 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 30 U ml−1 IL-2 (Roche). After 2 days, cells were washed and transferred to uncoated plates. On day 5, 1 × 10^6 activated OT1 T cells were incubated with 1 × 10^6 EL4 or GzmB activity nanosensors for 2 h at 37 °C and stained for GzmB using anti-mouse GzmB (clone: NG2B, eBioScience) and the Intracellular Fixation & Permeabilization Buffer Set (eBioScience, 88-8824-00). To measure GzmB activity inside target cells, we co-activated OT1 CD8+ T cells with EL4 and GzmB-targeting activity nanosensors.

Skin graft scoring and survival analysis. Skin grafts were qualitatively scored on a scale ranging from 0–4 according to established protocol by the Emory Transplant Center. Scoring involved direct observation and palpation of the graft and surrounding tissue. A score of 0 is given when a graft has been fully rejected and the site is completely red or black. A score of 1 is given for a single red ulcer comprising less than 25% of the graft area. Other reasons for a score of 3 might be scabbed over or scabbed. A score of 4 is given for a perfect-appearing skin graft. If donor was of BALB/c origin, the skin graft score of a score of 4 would be quite typical and slightly velvety, with hair growing from the graft and no imperfection noted. A score of 3 is for grafts that are good but not perfect in appearance, such as a graft that might otherwise merit a 4 but for a single red ulcer comprising less than 25% of the graft area. Other reasons for a score of 3 might be fain scabdness over the ear skin graft or slight hardening at the edges of a tail skin graft. A score of 2 is given for grafts that are half necrotic. For example, half of the graft area may be covered by red ulcers or scaly, red, thickened skin, but half of the graft still consists of viable skin. Grafts that have shrunk to 50% of their original size receive a score of 2. A score of 1 is given for grafts that are nearly completely necrotic but still have some small areas of viable skin. Grafts with a score of 1 will typically be totally necrotic within one week. A score of 0 is given when a graft has been fully rejected and the transplanted skin is completely necrotic. For Kaplan–Meier survival analysis, a skin graft was considered rejected when its score was below 1.
Pharmacokinetic studies. Mice bearing skin grafts were administered with nanosensors (20µg) or peptides (10 nmol) labelled with VivoTag S-750 (VT750; PerkinElmer). Nanosensors and peptides were labelled with VT750 (3:1 molar ratio) using NHS chemistry per manufacturer’s protocol. For organ biodistribution, whole mice were imaged with IVIS Spectrum CT Imaging System (PerkinElmer) while excised organs were imaged with Odyssey CLx Imaging System (LI-COR) 24h after administration. For urine pharmacokinetics, whole mice were imaged with IVIS Spectrum CT Imaging System (PerkinElmer) at 90 min post administration. To track cleared fragments in vivo GzmB clearance on day 7 post-transplant, VT750-labelled activity nanosensors (10nmol by peptide) were administered to skin graft mice. At 90 minutes post administration, the whole mouse was imaged with IVIS Spectrum CT Imaging System (PerkinElmer) to analyse the extend of fluorescence from the bladder. Major organs were then excised and homogenized using Lysing Matrix A Tubes and FastPrep24 Homogenizer (MP Biomedicals). Tissue homogenates were transferred to a 96-well plate and then imaged with the Odyssey CLx Imaging System (LI-COR).

GzmB activity imaging. GzmB substrate peptides with non-natural amino acids for manual labelling (kGgsEjFDSGGSsP[RA]c) was purchased from Genscript. After the peptides were coupled to IONPs, the NIR dye IRDye-800CW (LI-COR) was coupled to L-propargylglycine (PRA) via copper catalyzed click chemistry45. The NIR quencher was then coupled to the N-terminal lysine via traditional NHS chemistry according to the manufacturer’s protocol to generate the NIR GzmB activity probes. The extent of successful quenching and enyzmatic activation were analysed in vitro by Alexa Fluor analysis and Lipoic acid imaging upon activation of recombinant GzmB (Peptotech). For in vivo GzmB activity imaging study, NIR GzmB activity probes (10 nmol by peptide) were administered to skin graft mice. Major organs were excised at 24h and imaged with the Odyssey CLx Imaging System (LI-COR).

GzmB characterization in skin graft mouse model. For histological analysis, tissues were collected from skin graft mice on POD 7. All tissues were fixed in 4% paraformaldehyde (EMS) overnight at 4°C, washed 3 times with PBS and stored in 70% ethanol (VWR) until paraffin embedding, sectioning and staining for GzmB and CD8 (Winship Pathology Core). To verify flow cytometry analysis, 1 x 10⁶ spleenocytes or lymphocytes were isolated from skin graft mice on PODs 5, 7 and 9 were reconstituted with 1 x 10⁶ BALB/c spleenocytes for 4h at 37°C before staining for GzmB using anti-mouse GzmB (clone: NGZB, eBioScience) and an Intracellular Fixation & Permeabilization Buffer Set (eBioScience).

Urinary prediction of acute rejection in skin graft mice. All urineanalysis experiments were done in paired setup. Before (POD – 4) and at various time points after transplantation, skin graft mice were administered with FITC-labelled activity nanosensors (10 nmol by peptides). Mice were placed over 96-well polystyrene plates surrounded by an upper cylindrical sleeve covered by a weighted petri dish to prevent animals from jumping out of the cylinder. Animals were left to urinate for 90 min before urine samples were collected. FITC in urine was purified by a magnetic separation assay using Dynabeads (Thermo, 65501) coated with anti-FITC antibody (GeneTex, GTX10257) according to manufacturer's protocol. Fluorescent signals were measured with Cytation 5 Plate Reader (Biotek). Concentrations of FITC from urine samples were calculated using a free FITC ladder and normalized with urine volume to obtain percent injected dose. To account for batch-to-batch differences in nanosensors, the percent of injected dose is normalized by the average and variance to pre-transplant signals and plotted as normalized urine fluorescence.

In vivo CD8+ T cell depletion. For CD8+ depletion study, mice were given anti-mouse CD8 (clone: 53–6.7, BioXCell) for 3 consecutive days following with booster shots every 3 days after. Flow cytometry analysis of splenocytes and lymphocytes involved in acute renal allograft rejection. Kidney Int. 47, 70–77 (1995).

Administration of immunosuppression. For drug response study, allotroge mice were given either tacrolimus (2 mg.kg⁻¹, intraperitoneal; Prograf) or saline every day starting from POD – 1 until POD 7. Mice were given two more injections on PODs 9 and 11.

Immunogenicity study. On POD 0, C57BL/6 mice were inoculated intraperitoneally with 2000 µL of GzmB peptide (10 nmol), GzmB nanosensors (10 nmol by peptide), GzmB nanosensors (10 nmol by peptide, 100 µl) + complete Freund adjuvant (Sigma; Sigma), chicken myoglobin (100 µg), chicken myoglobin (100 µg) + CFA (100 µg) and saline. Blood was collected via retroorbital blood draw into serum collection tube (CAPIJECt) and centrifugated for 5 min at 3500g. Serum samples were collected and stored at –80°C until analysis. Total IgG levels in serum were determined using an Easy-Titer Mouse IgG Assay Kit (Thermo).

Software and statistical analysis. Graphs were plotted and appropriate statistical analyses were conducted using GraphPad Prism (P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; central values depict the means, and error bars depict s.e.m.). Quantification of histological images was performed on Image (NIH). Whole-mouse fluorescent data were analysed using Living Image (PerkinElmer). Whole-organ fluorescent data were analysed using Image Studio (LI-COR). Flow cytometry data were analysed using FlowJo X (FlowJo, LLC). Power analyses were performed using G*Power 3.1 (HHUD).

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability All data supporting the findings of this study are available within the manuscript and its Supplementary Information. Raw data are available from the corresponding authors.

Received: 30 January 2018; Accepted: 16 January 2019; Published online: 18 February 2019

References
1. Mas, V. R., Mueller, T. F., Archer, K. J. & Maluf, D. G. Identifying biomarkers as diagnostic tools in kidney transplantation. Expert. Rev. Mol. Diagn. 11, 183–196 (2011).
2. Gwinn, W. Renal transplant rejection markers. World J. Urol. 25, 445 (2007). Check all relevant references.
3. Cornell, L. D., Smith, R. N. & Colvin, R. B. Kidney transplantation: mechanisms of rejection and acceptance. Annu. Rev. Pathol. Mech. Dis. 3, 189–220 (2008). Check all relevant references.
4. Nankivel, B. J. & Alexander, S. I. Rejection of the kidney allograft. N. Engl. J. Med. 363, 1451–1462 (2010).
5. Sjöblom, C. & Nilsson, C. O. Early versus late acute rejection episodes in renal transplantation. Transplantation 75, 204 (2003).
6. Moreau, A., Varey, E., Anegon, I. & Caturelli, M.-C. Effector mechanisms of rejection. Cold Spring Harb. Perspect. Med. 3, a015461 (2013).
7. Furness, P. N., Taul, N. & Converging of European Renal Transplant Pathology Assessment Procedures (CERTPAF Project). International variation in the interpretation of renal transplant biopsies: report of the CERTPAF Project. Kidney Int. 60, 1998–2012 (2001).
8. Piovesan, A. C. et al. Multifocal renal allograft biopsies: impact on therapeutic decisions. Transplant. Proc. 40, 3397–3400 (2008).
9. Jaffa, M. A. et al. Analyses of renal outcome following transplantation adjusting for informative right censoring and demographic factors: a longitudinal study. Transplant. Rev. 32, 691–698 (2010).
10. Josephson, M. A. Monitoring and managing graft health in the kidney transplant recipient. Clin. J. Am. Soc. Nephrol. 6, 1774–1780 (2011).
11. Vlamink, I. D. et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. Sci. Transl. Med. 6, 241ra77–241ra77 (2014).
12. Choy, J. C. Granzymes and perforin in solid organ transplant rejection. Curr. Opin. Organ Transplant. 17, 567–576 (2010).
13. Choy, J. C. Granzymes and perforin in solid organ transplant rejection. Curr. Opin. Organ Transplant. 17, 567–576 (2010).
14. Choy, J. C. Granzymes and perforin in solid organ transplant rejection. Curr. Opin. Organ Transplant. 17, 567–576 (2010).
15. Kummer, J. A. et al. Expression of granzyme A and B proteins by cytotoxic lymphocytes involved in acute renal allograft rejection. Kidney Int. 47, 70–77 (1995).
16. Suthanthiran, M. et al. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. N. Engl. J. Med. 369, 20–31 (2013).
17. Simon, T., Opedz, G., Wiesel, M., Ott, R. C. & Süss, C. Serial peripheral blood perforn and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. Am. J. Transplant. 3, 1121–1127 (2003).
18. Calafiore, R. & Basta, G. Clinical application of microencapsulated islets: actual perspectives on progress and challenges. Adv. Drug Deliv. Rev. 67, 84–92 (2014).
19. Li, B. et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N. Engl. J. Med. 344, 947–954 (2001).
20. Sun, J. et al. A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine regulator modifier A is present in cytotoxic lymphocytes. J. Biol. Chem. 271, 27802–27809 (1996).
21. Edington, L. E., Verdoes, M. & Bogoy, M. Functional imaging of proteases: recent advances in the design and application of substrate-based and activity-based probes. Curr. Opin. Chem. Biol. 15, 798–805 (2011).
22. Flavin, J. & Activity-based profiling of proteases. Annu. Rev. Biochem. 83, 249–273 (2014).
23. Konishi, M. et al. Imaging granzyme B activity assesses immune-mediated myocarditis. Circ. Res. 117, 502–512 (2015).
53. Maeda, H., Wu, J., Sawa, T., Matsumura, Y. & Horii, K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284 (2000).

54. Fodor, G. et al. Targeted nanoparticles containing the proresorbing peptide Ac2-26 protect against advanced atherosclerosis in hypercholesterolemic mice. Sci. Transl. Med. 7, 275ra20 (2015).

55. Wilhelm, S. et al. Analysis of nanoparticle delivery to tumours. Nat. Rev. Mater. 1, 16014 (2016).

56. Rani, S. H. et al. Renal clearance of nanoparticles. Nat. Biotechnol. 25, 1165–1170 (2007).

57. Louny, A. et al. The Banff 2015 Kidney Meeting Report: current challenges in rejection classification and prospects for adopting molecular pathology. Am. J. Transplant. 17, 28–41 (2017).

58. Vincenti, F., Jensik, S. C., Filo, R. S., Müller, J. & Pirsch, J. A long-term comparison of tacrolimus (FK506) and cyclosporine in kidney transplantation: evidence for improved allograft survival at five years. Transplantation 73, 775–782 (2002).

59. Vincenti, F. et al. Belatacept and long-term outcomes in kidney transplantation. N. Engl. J. Med. 374, 333–343 (2016).

60. Friedman, G. & Haraldseth, O. Morphometric and charge selectivity in the mouse after exposure to glucosaminoglycan-degrading enzymes. J. Am. Soc. Nephrol. 14, 1756–1765 (2003).

61. Hsauyry, P. et al. The inflammatory mechanisms of allograft rejection. Immunol. Cell Biol. 94, 0054 (2016).

62. Wood, K. J. & Goto, R. Mechanisms of rejection. Curr. Persp. Transplant. 93, 1–10 (2012).

63. Stauquini, F. I. et al. Vascular ligand-receptor mapping by direct combinatorial selection in cancer patients. Proc. Natl Acad. Sci. USA 108, 18637–18642 (2011).

64. Hua, S. Targeting sites of inflammation: intercellular adhesion molecule-1 as a target for novel inflammatory therapies. Front. Pharmacol. 4, 127 (2013).

65. Kwon, E. J., Dудани, J. S. & Bhata, S. N. Ultrasensitive tumour-penetrating nanosensors of protease activity. Bio. Med. Eng. 1, 0054 (2017).

66. Han, D. et al. Assessment of cytotoxic lymphocyte gene expression in the peripheral blood of human islet allograft recipients: elevation precedes clinical evidence of rejection. Diabetes 53, 2281–2290 (2004).

67. Jaffer, F. A. & Weissleder, R. Molecular imaging in the clinical arena. JAMA 293, 855–862 (2005).

68. Weissleder, R., Tung, C.-H., Mahmod, U. & Bogdanov, J. In vivo imaging of tumours with protease-activated near-infrared fluorescent probes. Nat. Biotechnol. 17, 375–378 (1999).

69. Olson, E. S. et al. Activatable cell penetrating peptides linked to nanoparticles as dual probes for in vivo fluorescence and MR imaging of proteases. Proc. Natl Acad. Sci. USA 107, 4311–4316 (2010).

70. Sugahara, K. N. et al. Tissue-penetrating delivery of compounds and nanoparticles into tumors. Cancer Cell. 16, 510–520 (2009).

71. Horri, S. S. & Gambhir, S. S. Mathematical model identifies blood biomarker-based early cancer detection strategies and limitations. Sci. Transl. Med. 3, 103ra16–103ra11 (2011).

72. Lutz, A. M., Willmann, J. K., Cochran, F. V., Ray, P. & Gambhir, S. S. Cancer screening: a mathematical model relating secreted blood biomarker levels to tumor sizes. PLoS Med. 5, e170 (2008).

73. Dharmadhikara, V. R., Kwon, C. & Stevens, G. Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. Am. J. Kidney. Dis. 40, 221–226 (2002).

74. Kaplan, B., Schold, J. & Meier-Kriesche, H.-U. Poor predictive value of serum creatinine for renal allograft loss. Am. J. Transplant. 3, 1560–1563 (2003).

75. Sioucum, J. L., Heung, M. & Pennathur, S. Marking renal injury: can we move beyond serum creatinine? Trans. Res. 159, 277–289 (2012).

76. Haase, M. et al. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am. J. Kidney. Dis. 54, 1012–1024 (2009).

77. Mishchak, H. et al. Recommendations for biomarker identification and qualification in clinical proteomics. Sci. Transl. Med. 2, 64ps42–64ps42 (2010).

78. Prendergast, M. B. & Gaston, R. S. Optimizing medication adherence: an ongoing opportunity to improve outcomes after kidney transplantation. Clin. J. Am. Soc. Nephrol. 5, 1303–1311 (2010).

79. Allagandage, G. J. et al. Infectious complications after kidney transplantation: current epidemiology and associated risk factors. Clin. Transplant. 20, 401–409 (2006).

80. Sellars, S. J. et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am. J. Transplant. 12, 3880–3892 (2012).

81. Palmacci, S. Synthesis of polysaccharide covered superparamagnetic oxide colloids. US patent 5,262,176 (1993).

82. Presolski Stanislav, I., Hong Vu, Phong. & Finn, M. G. Copper-catalyzed azide–alkyne click chemistry for bioconjugation. Curr. Protoc. Chem. Biol. 3, 153–162 (2011).
Acknowledgements
This work was funded by an NIH Director’s New Innovator Award DP2HD091793 awarded to G.A.K. and National Institutes of Health U01 AI132904 awarded to A.B.A. Q.D.M. is supported by the NSF Graduate Research Fellowships Program (Grant No. DGE-1650044). D.V.M. is supported by National Institutes of Health F30 award number DK109665. B.A.H is supported by the National Institutes of Health GT BioMAT Training Grant under Award Number 5T32EB006343. G.A.K. holds a Career Award at the Scientific Interface from the Burroughs Wellcome Fund. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions
Q.D.M., D.V.M., A.B.A. and G.A.K. conceived of the idea, designed experiments, and interpreted results. Q.D.M., D.V.M., J.A.K., C.M.S., O.M.D. and B.A.H. carried out the experiments. Q.D.M., D.V.M., B.A.H., A.B.A. and G.A.K. wrote the manuscript.

Competing interests
Q.D.M., D.V.M., A.B.A. and G.A.K. are listed as inventors on a patent application pertaining to the results of the paper. G.A.K. is co-founder of and serves as consultant to Glympse Bio, which is developing products related to the research described in this paper. This study could affect his personal financial status. The terms of this arrangement have been reviewed and approved by Georgia Tech in accordance with its conflict of interest policies.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41551-019-0358-7.
Reprints and permissions information is available at www.nature.com/reprints.
Correspondence and requests for materials should be addressed to A.B.A. or G.A.K.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
© The Author(s), under exclusive licence to Springer Nature Limited 2019
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- Confirmed

  - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
  - An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
  - A description of all covariates tested
  - A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
  - A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
  - For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
  - For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
  - For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
  - Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
  - Clearly defined error bars
  - State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Fluorescent data from microplate were collected with Gen5 2.07 (Biotek). Whole-mouse fluorescent data were collected with Living Image 4.4.5 (PerkinElmer). Whole-organ fluorescent data were collected with Image Studio 5.2 (LI-COR). Flow-cytometry data were collected with BD FACSDIVA v8 (BD Biosciences).

Data analysis

Quantification of histological images was performed on ImageJ mod Fiji 1.51n. Whole-mouse fluorescent data were analysed using Living Image 4.4.5 (PerkinElmer). Whole-organ fluorescent data were analysed using Image Studio 5.2 (LI-COR). Flow-cytometry data were analysed using FlowJo X (FlowJo, LLC).

Graphs were plotted and appropriate statistical analyses were conducted using GraphPad Prism 6.05 (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; all error bars depict s.e.m.).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data supporting the findings of this study are available within the manuscript and its Supplementary Information. Raw data is available from the corresponding authors.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences  Behavioural & social sciences  Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Power analyses were performed (G*Power 3.1.9.2) to determine sample size, taking into account the effect size (dz) from preliminary data. For in vivo urinalysis experiments, using a conservative approach (dz between 1 and 2.2), we estimated that our experiments required a minimum of 4–9 animals to reach statistical power equal to or greater than 0.8. |
| Data exclusions | No data were excluded from the analyses. |
| Replication | All attempts at replication were successful. Furthermore, to ensure reproducibility of the experimental findings, in addition to replicating the experiments with biological replicates, we often used several experimental methods to confirm a particular finding. For example, GzmB expression in the skin-graft model was confirmed by both flow cytometry and immunohistochemistry. Similarly, the ability of nanosensors to detect the early onset of rejection was confirmed qualitatively with NIR fluorescent imaging and quantitatively with an urine assay. |
| Randomization | Animals with the same age and sex were allocated into experimental groups randomly. |
| Blinding | The majority of experiments (nanosensors cleavage assay, T-cell assays, NIR fluorescence imaging, and urine assays) were done with knowledge of group allocations during data collection. This was because skin allografts are from C57BL/6 (black skin) whereas isografts are from BALB/c (white skin). Regardless, each mouse has an ear tag to facilitate downstream identification. All downstream analyses were done without knowledge of group allocation. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| --- | --- |
| [ ] | Unique biological materials |
| [ ] | Antibodies |
| [ ] | Eukaryotic cell lines |
| [ ] | Palaeontology |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |

Methods

| n/a | Involved in the study |
| --- | --- |
| [ ] | ChIP-seq |
| [ ] | Flow cytometry |
| [ ] | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials
FITC-labeled custom peptides were synthesized by Tufts University Peptide Core. Custom peptides with fluorophore-quencher pair for in vitro cleavage assays were synthesized by CPC Scientific. All custom materials can be purchased directly from these commercial sources.
### Antibodies

| Antibodies used | For plate activation of splenic CD8+ T cells: anti-mouse CD3e (Clone: 145-2C11, BD), anti-mouse CD28 (Clone: 37.51, BD). For flow cytometry analysis: anti-mouse GzmB (Clone: NGZB, eBioScience), anti-mouse CD3 (clone: 17A2, Biolegend), anti-mouse CD4 (clone: RM4-5, Biolegend), anti-mouse CD8 (clone: KT15, Serotec). All antibodies for flow analysis were used at 100x dilution. For histological analysis: anti-mouse or human GzmB (1/100 dilution, polyclonal, product #ab4059, Abcam), anti-mouse CD8a (1/500 dilution, polyclonal, product #ab203035, Abcam). |
| --- | --- |

| Validation | Antibody staining was compared to manufacturer's QC data. Furthermore, antibodies were validated by inclusion of positive and negative controls in experiments, as well as by using orthogonal methods such as ELISA. |

### Eukaryotic cell lines

| Policy information about cell lines |  |
| --- | --- |
| **Cell line source(s)** | EL4 (ATCC), E.G7-OVA (ATCC) |
| **Authentication** | E.G7-OVA cells express OVA antigen, confirmed by staining of anti-mouse MHC class I Kb-SIINFEKL (Clone: 25-D1.16, Biolegend). Staining on EL4 cells showed no expression of this marker. |
| **Mycoplasma contamination** | All cell lines were tested negative for mycoplasma contamination. |
| **Commonly misidentified lines** (See ICLAC register) | No commonly misidentified cell lines were used in this study. |

### Animals and other organisms

| Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research |  |
| --- | --- |
| **Laboratory animals** | OT-1 mice are transgenic mice in which their T cells have been genetically engineered to recognize only one specific antigen, instead of a broad range of antigens that T cells in a wild-type mouse would normally recognize. The antigens are chicken ovalbumin (OT-1). T cells from OT1 mice are used as the model of antigen specific T cell killing. We used C57BL/6 mice as recipients for grafts from BALB/c mice because their MHC molecules are mismatched, and therefore, promotes T cell mediated acute rejection. All mice used in this study were female and 6–8 weeks old. |
| **Wild animals** | The study did not involve wild animals. |
| **Field-collected samples** | The study did not involve samples collected from the field. |

### Flow Cytometry

| Policy information about plots | Confirm that: |
| --- | --- |
| | ☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC). |
| | ☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers). |
| | ☑ All plots are contour plots with outliers or pseudocolor plots. |
| | ☑ A numerical value for number of cells or percentage (with statistics) is provided. |

| Policy information about methodology |  |
| --- | --- |
| **Sample preparation** | For flow-cytometry experiments, single cells were isolated from spleens and lymph nodes of mice using standard protocols. Tissues were dissociated between frosted glass slides (VWR 48332-004) and filtered with a 40-μm cell strainer (VWR 10199-654). RBC were lysed with RBC lysis buffer (VWR 420301-BL). Cell count and viability of the final single-cell suspension were determined by Trypan Blue staining with Countess II FL Cell Counter (Thermo). |
| **Instrument** | Data for the flow-cytometry experiments were collected using BD LSR Fortessa (BD Biosciences). |
| **Software** | Flow-cytometry data were collected with BD FACSDiva v8 (BD Biosciences). Analyses were done in FlowJo X (FlowJo, LLC). |
| **Cell population abundance** | No sorting experiments were done in these studies. For flow-cytometry experiments, each sample contained 10^6 viable cells. After staining, 3x10^4S total events were recorded for each sample before statistical tests were performed. |
| **Gating strategy** | A conservative gate was drawn on the FSC-A x SSC-A plot. Single cells were then gated using FSC-W x FSC-H plot. Live cells were
Gating strategy gated by staining with Live/Dead Dye (Thermo). Subsequent gating was then to select for the cell population of interest. An example of gating strategy to select for viable CD8 T cells is provided in Supplementary Fig. 20.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.